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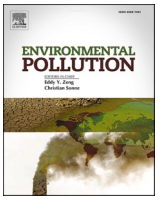

Assessment of acrylamide exposure in Spain by human biomonitoring: Risk and predictors of exposure☆

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ABSTRACT

Acrylamide (AA), a chemical compound currently classified as "reasonably anticipated to be a human carcinogen", is formed through the Maillard reaction in processed carbohydrate-rich foods and is also present in tobacco smoke. The primary sources of AA exposure in the general population are dietary intake and inhalation. Within a 24-h period, humans eliminate approximately 50% of AA in the urine, predominantly in the form of mercapturic acid conjugates such as N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(2-carbamoyl-2- hydroxyethyl)-L-cysteine (GAMA3), and N-acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine (AAMA-Sul). These metabolites serve as short-term biomarkers for AA exposure in human biomonitoring studies. In this study, we analysed first-morning urine samples from the adult population (aged 18–65 years) residing in the Valencian Region, Spain, (n = 505). AAMA, GAMA-3 and AAMA-Sul were quantified in 100% of the analysed samples, with geometric means (GM) of 84, 11 and 26 μg L^{-1}, respectively, while the estimated daily intake of AA in the studied population ranged from 1.33 to 2.13 μg·kg-bw^{-1}·day^{-1} (GM). Statistical analysis of the data indicated that the most significant predictors of AA exposure were smoking and the amount of potato fried products and, biscuits and pastries consumed last 24 h. Based on risk assessment approaches conducted, the findings suggest that exposure to AA could pose a potential health risk. Therefore, it is crucial to closely monitor and continuously evaluate AA exposure to ensure the well-being of the population.

1. Introduction

Acrylamide (AA), a low-molecular weight compound, can be found abundantly in carbohydrate-rich foods as a result of the Maillard reaction, which occurs when high temperatures (greater than 120 °C) are applied and involves the interaction between free amino-acids, with a particular emphasis on asparagine, and reducing sugars like glucose and fructose (Stadler et al., 2002; Mottram et al., 2002). The European Food Safety Authority (EFSA) provides an estimation of the daily intake of AA in the European population, ranging from 0.4 to 1.9 μg AA·kg-bw^{-1}·day^{-1}, and highlights the significant contributors from the consumption of coffee, potato fried products, bread, and biscuits (EFSA, 2015). Furthermore, it is worth noting that AA is not only present in carbohydrate-rich foods, but is also a constituent of tobacco smoke (McAdam et al., 2015) and finds applications in various sectors of the chemical industry, such as cosmetics and food packaging (EFSA, 2015). Consequently, the exposure pathways for humans encompass ingestion, inhalation and dermal uptake; however, among the non-smoker population, ingestion emerges as the predominant route of exposure.

Upon entry into the human body, AA undergoes rapid absorption and is subjected to metabolism in two distinct phases. In the initial phase, AA is subjected to biotransformation mediated by cytochrome P450 2E1 (CYP2E1), resulting in the formation of glycidamide (GA). Subsequently, in the second phase, both AA and GA undergo a chemical reaction known as Michael addition with glutathione (GSH) transferases, leading to the production of GSH conjugates. These conjugates can further undergo metabolism to generate specific mercapturic acids, N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA) originating from AA, as well as N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA-3) and N-acetyl-S-(1-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA-2)

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derived from GA. Subsequently, these mercapturic acids are eliminated from the body through urinary excretion. It is worth noting that in humans, AAMA can undergo oxidation to form its corresponding sulfoxide compound, N-acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine (AAMA-Sul) (Zhang et al., 2015).

Experimental studies conducted on animals have provided substantial evidence indicating the neurotoxic, carcinogenic, mutagenic, genotoxic, and immunotoxic effects of AA (Eisenbrand, 2020). However, current epidemiological and toxicological investigations carried out in humans have not consistently corroborated these effects thus far. Nevertheless, due to the potential risks posed to human health, AA has been classified as a "probable carcinogen to humans" (Group 2 A) by the International Agency for Research on Cancer (IARC) (IARC, 1994) and as "reasonably anticipated to be a human carcinogen" in the 15th Report on Carcinogens (2021) by the US National Toxicology Program. In 2015, the EFSA issued a scientific opinion on AA, expressing concerns regarding its carcinogenic effects and the lack of consistent decline in AA levels over the years, suggesting a potential health risk associated with human exposure to AA (EFSA, 2015). Consequently, the European Commission implemented Regulation 2017/2158 on November twentieth, 2017, which established mitigation measures and benchmark levels for reducing AA exposure through dietary intake (European Commission, 2017). Additionally, the European Parliament enacted Regulations 1223/2009 and 10/2011, which prohibited and restricted the use of AA in cosmetic products and food-contact materials, respectively (EFSA, 2015). Furthermore, the Human Biomonitoring for Europe (HBM4EU) initiative included AA as one of the prioritized substances in its second round of assessments (HBM4EU, 2020). By employing human biomonitoring (HBM), the HBM4EU initiative enables the evaluation of internal exposure to AA through all potential pathways, providing a more representative assessment than the commonly used estimated daily intakes (EDIs) based on food levels and consumption data when assessing the risk associated with AA exposure (EFSA, 2015; F. Fernández et al., 2022a).

The determination of internal exposure to AA can be accomplished by analysing AA metabolites present in blood or urine samples (Luoro et al., 2019). In blood, both AA and GA form adducts with haemoglobin through Michael addition, which are considered suitable biomarkers for assessing long-term exposure (Hartmann et al., 2008). However, urine is generally the preferred specimens for AA biomonitoring because of approximately 50% of its urinary metabolites are eliminated within the first 24 h following exposure (Kocadağli and Gökmen, 2016). Furthermore, these urinary metabolites are stable compounds that can be quantified with high specificity and sensibility, making them appropriate for the determination of recent AA exposure (HBM4EU, 2020). The primary urinary metabolite of AA is AAMA, which is typically the most abundant, followed by AAMA-Sul and GAMA-3. On the other hand, GAMA-2 levels are usually extremely low and therefore not commonly used as a biomarker for AA exposure. Additionally, it is worth noting that urine is a non-invasive matrix that can be easily obtained in large volumes (F. Fernández et al., 2022a). In the context of risk assessment, Biomonitoring Equivalents (BEs) have been proposed as screening tools for interpreting HBM data. BEs are defined as the concentration of a chemical or its metabolites in biological sample that are consistent with an existing health-based exposure guidance value (Hays and Aylward, 2009). Hays and Aylward (2008) estimated that the BE value for urinary AAMA concentration to be 13 μg AAMA per liter of urine and 16 μg AAMA per gram of creatinine, based on volume and creatinine levels, respectively.

Although there are studies reporting AA exposure worldwide, in recent years, only a few of them are conducted through urine. These studies have particularly focused on vulnerable groups, such as children, adolescents or women (Heudorf et al., 2009; Choi et al., 2019; Schwedler et al., 2021; Mojska et al., 2016). However, in terms of the adult population, they have primarily aimed to compare urinary AA levels between smokers (including users of electronic and/or tobacco cigarettes) and non-smokers (Frigerio et al., 2020; Kenwood et al., 2022). As a result, the understanding of internal AA exposure in the adult population is still limited. Additionally, the production of studies that show determinants of exposure is awfully poor. In the Spanish context, only two studies have investigated AA exposure, both of which focused on vulnerable populations, specifically lactating mothers and children (F. Fernández et al., 2022a, 2022b). Unfortunately, there is currently no data available regarding internal AA exposure in the adult population of Spain.

Therefore, the main objective of the present study is to examine the levels of urinary AA metabolites in occupational adult population residing in the Valencian Region of Spain. The aim is to enhance our understanding of the risk associated with AA exposure in the adult population and provide insight into the current levels of AA exposure. While other subgroups of the Spanish population have been previously analysed, the existing information is insufficient to determine the extent of AA exposure and assess the associated risks experienced by the Spanish population as a whole. Furthermore, the study also assesses the risk linked to the occurrence of AA in urine and explores potential predictors of AA exposure.

2. Materials and methods

2.1. Study population and recruitment of participants

This study was conducted as part of the BIOMOVAL project, a three-year (2021–2023) HBM cross-sectional study on the occupational adult population residing in the Valencian Region of Spain. The project was initiated and supported by the Directorate General of Public Health and Addictions, in collaboration with FISABIO. To ensure the representativeness of the population, the recruitment process followed a stratified approach based on the distribution of participants according to sex, age (categorized into four age intervals), and location (considering the population size in each province). The stratified recruitment aimed to closely adhere to these guidelines by randomly selecting participants to fill each stratum, in line with the predetermined selection criteria outlined in the project design. These selection criteria were carefully established to ensure the inclusion of relevant participants.

- Aged between 18 and 65 years old
- Living in the Valencian Region at least 5 years
- Not to have a urinary disease, a catheter or other reasons which hinder for urinary properly according to the questionnaire.

During 2021, a total of 548 subjects, consisting of 285 males and 263 females, were recruited for the study in select Prevention Health Centres affiliated with the Association of Occupational Risk Prevention Services of the Valencian Region (SERPRECOVA), in collaboration with the University of Jaume I (UJI).

2.2. Data and sample collection

Finally, 540 out of 548 subjects who agreed to participate in the study provided a first-morning urine sample, which was collected in a 100-mL polypropylene container and rapidly transported within 24 h of collection using isothermal packaging. The packaging ensured refrigerated transport at 4 °C to prevent compound degradation until the samples were stored in 10-mL aliquots at −80 °C in the Biobank for Biomedical Research and Public Health of the Valencian Region (IBSP-CV Biobank, Spain; PT13/0010/0064).

Specific gravity (SG) and urinary creatinine (Cre) levels were measured to account for variations in urine dilution among the samples. SG values were determined by measuring the Brix grades of urine samples, prior to storage, using a refractometer from Lab Logistics Group GmbH (Meckenheim, Germany), while Cre concentrations were obtained using the Jaffé's reaction method, using a biochemical parameter

analyser called KROMA from Linear Chemicals (Barcelona, Spain). Cre levels were used to exclude urine samples with abnormal Cre concentrations, i.e., outside the range of 30–300 mg dL^{-1} (Barr et al., 2005), due to normal values for SG have not still been described and both are directly correlated since depend on the hydration status of the samples.

2.3. Ethical and confidentiality

BIOMOVAL project was approved by the Ethics Committee for Research of the General Directorate of Public Health and Research of the Foundation for the Promotion of Health and Biomedical Research of the Valencian Region. Prior to data and sample collection, all participants provided written informed consent. All personal data were handled, transferred, and analysed in a secure environment and will be retained only for the necessary processing period. The processing of data adhered to all national and EU legal and ethical guidelines, specifically the General Data Protection Regulation.

2.4. Determination of urinary acrylamide metabolites

Details of chemicals, standards, sample preparation, chromatographic conditions, and quality assurance/quality control (QA/QC) criteria used to analyse AAMA, GAMA-3 and AAMA-Sul in the urine samples of the present study have been comprehensively described previously by F. Fernández et al. (2022a). Briefly, a "dilute and shoot" method using 100 μL of urine followed by determination by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) was employed.

2.5. Statistical analysis

The levels of urinary AA metabolites were adjusted by volume ($\mu g \cdot L^{-1}$), SG ($\mu g \cdot L^{-1}$), and urinary Cre levels ($\mu g \cdot g\ Cre^{-1}$) and were summarized using the most relevant statistic descriptors: detection frequency (DF, %), arithmetic mean (AM), geometric mean (GM), 25th, 50th, 75th and 95th percentiles (P25, P50, P75, and P95), minimum, maximum values, and standard deviation (SD). The adjustment of urinary AA metabolites concentrations by SG was carried out using the formula reported by Kuiper et al. (2021). The most relevant information from the questionnaires was extracted and qualitative variables were described by absolute frequencies and percentages, and quantitative variables by their median, minimum and maximum values. BMI was calculated by the weight and height of the people. Regarding smoking status, since the questionnaire did not include the option of passive smoking, individuals who were not current smokers but lived with a smoker were identified as passive smokers. Additionally, certain foods were categorized into groups, and individuals who consumed any amount of fried potato products, biscuits, or pastries in the last 24 h were classified as consumers of those items within the last 24 h.

Currently, there is no consensus about which method is preferable to correct urinary levels of chemicals due to the different hydration states of the samples (HBM4EU, 2019). However, the use of SG values as a correction method is interesting, especially since Kuiper et al. (2021) concluded that they are likely preferable to the Cre levels as a measure of urinary dilution in studies of urinary biomarkers of chemicals. Furthermore, Qian et al. (2021) already used the SG values as a correction variable of urinary concentration of multiple volatile organic compound (VOC) metabolites. Therefore, in the present study, although the urinary AA metabolites levels and all of the indicators are shown using the three correction methods (adjusted by volume, by Cre and by SG), we were more focused in the correction by SG.

For data analysis, IBM SPSS statistics software was used. The Spearman's correlation test was performed to determine the bivariate correlations among the three urinary AA metabolites individually. Raw urinary AA metabolites concentrations ($\mu g \cdot L^{-1}$) were transformed to $nmol \cdot L^{-1}$ and added together to obtain the sum of all of them ($\sum$UAAM). No dilution correction method, neither Cre nor SG, to convert the metabolite content in the regression model were used, but SG values were included as independent variable instead, following the suggestion of Barr et al. (2005). Next, in accordance with the HBM4EU recommendations (HBM4EU, 2019), a forward stepwise selection procedure based on the corrected Akaike information criterion (AICc) (Konishi and Kitagawa, 2008) was utilized to construct a linear regression model, aiming to explore the associations between $\sum$UAAM and the exposure variables (refer to Table 1). This approach was chosen due to the non-normal distribution of the raw data. To satisfy this requirement, the logarithm of the sum of $\sum$UAAM was used as the dependent variable and statistical significance was determined at a p-value <0.05. Following the construction of the regression model, several tests were conducted to verify the regression assumptions, including normality, non-autocorrelation, homoscedasticity, and non-multicollinearity. The Kolgomorov-Smirnov test, Durbin-Watson statistic, scatter plots, as well as tolerance and condition indexes, were employed for this purpose. Finally, to examine the relationship between the sum of urinary AA metabolites adjusted by SG and specific population groups, Mann-Whitney and Kruskal-Wallis tests were applied. These tests aimed to provide insights into the associations between the aforementioned metabolites and different population segments.

2.6. Estimated daily intake (EDI)

The Estimated Daily Intake (EDI) of a substance is calculated by EFSA taking into account the quantity of the substance found in food and beverages, as well as the daily consumption of these food and beverages items. When it comes to HBM studies, the EDI of a substance is determined using HBM data following the methodology established by EFSA. The EDI calculation serves as a first approximation to the intake dose of chemicals and because of a chemical could lead to the formation of more than one metabolite is recommended to use the sum of them (Gurusankar et al., 2017). Therefore, $\sum$UAAM (expressed as volume-based, SG-based and Cre-based concentrations) were transformed to EDIs of AA via the following equations (Heudorf et al., 2009).

$$EDI_{vol}\left(\frac{\mu g\ AA}{kg-bw\cdot day}\right)=\frac{\sum UAAM_{vol}\ x\ V_{24h}\ x\ MW_{AA}}{F_{UE}\ x\ BW} \quad (1)$$

$$EDI_{SG}\left(\frac{\mu g\ AA}{kg-bw\cdot day}\right)=\frac{\sum UAAM_{SG}\ x\ V_{24h}\ x\ MW_{AA}}{F_{UE}\ x\ BW} \quad (2)$$

$$EDI_{cre}\left(\frac{\mu g\ AA}{kg-bw\cdot day}\right)=\frac{\sum UAAM_{cre}\ x\ Cre_{24h}\ x\ MW_{AA}}{F_{UE}\ x\ BW} \quad (3)$$

where $\sum UAAM_{vol}$, $\sum UAAM_{SG}$, $\sum UAAM_{Cre}$ are the sum of the urinary molar concentrations of AAMA, AAMA-Sul and GAMA-3 ($\mu mol \cdot L^{-1}$ for EDI_{vol} and EDI_{SG}, or $\mu mol \cdot g\ Cre^{-1}$ for EDI_{cre}); V_{24h} is the estimated total urinary volume excreted within 24 h for adults ($1.65\ L \cdot day^{-1}$) obtained to the average between $1.7\ L \cdot day^{-1}$ for adult men and $1.6\ L \cdot day^{-1}$ for adult women (Aylward et al., 2011) due to equality in sex distribution; Cre_{24h} is the estimated daily urinary Cre excretion normalized by mean body weight (kg), age (years old) and height (cm) of the people under study ($1.48\ g\ Cre \cdot day^{-1}$), calculated as it is shown in Eq. 3 b by Mage et al. (2004); MW_{AA} is the molecular weight of AA ($71\ g\ mol^{-1}$); F_{UE} is the fraction of the ingested dose of AA eliminated as urinary mercapturic acids, which is set at 0.5 according to an approximation of excretion rates recollected by Kocadağli and Gökmen (2016); BW is the body weight of the participants.

2.7. Risk assessment

The health risks associated with the urinary AA levels can be assessed using two different approaches: reverse and forward dosimetry (Gurusankar et al., 2017), which are frequently used in HBM studies. Only the

Table 1
Socio-demographic characteristics, lifestyle and dietary habits of the studied population used for building the regression model (n = 505).

Population's descriptors		N (%)	Median (range)
Sex			
Male		267 (52.9)	–
Female		238 (47.1)	–
Age (years)		–	43 (18–65)
18–30		103 (20.4)	–
31–40		106 (21.0)	–
41–50		160 (31.7)	–
51–65		136 (26.9)	–
Socio-economic sector			
Farming		10 (2.0)	–
Construction		32 (6.3)	–
Industry		42 (8.3)	–
Services		421 (83.4)	–
Province			
Castellón		60 (11.9)	–
Valencia		265 (52.5)	–
Alicante		180 (35.6)	–
BMI ($kg{\cdot}m^{-2}$)		–	24.92 (16.94–56.30)
<18.50		11 (2.2)	–
18.50–24.99		247 (48.9)	–
≥25.0		247 (48.9)	–
Population's descriptors		N (%)	Median (range)
Educational level			
Any		14 (2.8)	–
Elementary school		111 (22.0)	–
Secondary school		122 (24.1)	–
University degree		258 (51.1)	–
Marital Status			
Single		105 (20.8)	–
Married		229 (45.3)	–
Separated		31 (6.2)	–
In pairs (living)		112 (22.2)	–
In pairs (not living)		28 (5.5)	–
Annual *per capita* income (euros, €)			
<14499		70 (13.9)	–
14500-19999		111 (22.0)	–
20000-34999		170 (33.6)	–
35000-59999		105 (20.8)	–
≥60000		49 (9.7)	–
Smoking status			
Non-smoker		306 (60.6)	–
Passive smoker		69 (13.7)	–
Smoker		130 (25.7)	–
Food related to AA exposure consumed last 24 h (grams)			
Precooked food		–	0 (0–500)
Biscuits and pastries		–	0 (0–450)
Bakery products		–	0 (0–1000)
Bread		–	100 (0–800)
Cereals		–	0 (0–375)
Snacks		–	0 (0–175)
Potato fried products		–	0 (0–875)
Coffee		–	50 (0–400)
People who consumed these food products last 24 h			
Biscuits and pastries	Yes	209 (41.4)	–
	No	296 (58.6)	–
Potato fried products	Yes	201 (39.8)	–
	No	304 (60.2)	–
People who used these culinary practises last 24 h			
Frying:	Yes	138 (27.3)	–
	No	366 (72.5)	–
	Missing data	1 (0.2)	–
Population's descriptors		N (%)	Median (range)
Grilling:	Yes	21 (4.2)	–
	No	483 (95.6)	–
	Missing data	1 (0.2)	–
Smoking:	Yes	31 (6.1)	–
	No	472 (93.5)	–
	Missing data	2 (0.4)	–
Baking:	Yes	186 (36.8)	–
	No	316 (62.6)	–
	Missing data	3 (0.6)	–
Roasting:	Yes	162 (32.1)	–
	No	337 (66.7)	–
	Missing data	6 (1.2)	–

risk of non-neoplastic effects was evaluated here because no clear evidences of neoplastic effects on humans have been described so far (EFSA, 2015).

2.7.1. Reverse dosimetry

In the reverse dosimetry approach, the EDI was compared to the lower confidence limit of the reference dose ($BMDL_{10}$) for neurotoxicity in rats (430 μg AA·kg-bw^{-1}·day^{-1}) as a health-based reference value by calculating the margin of exposure (MOE) following the next formula (EFSA, 2015).

$$\text{MOE (dimensionless)} = \frac{\text{BMDL}_{10}\left(\frac{\mu g\ AA}{kg-bw\cdot day}\right)}{\text{EDI}\left(\frac{\mu g\ AA}{kg-bw\cdot day}\right)} \quad (4)$$

EFSA suggests that MOEs values lower than 125 may indicate a human health concern due to non-genotoxic effects of AA (EFSA, 2015).

2.7.2. Forward dosimetry

In the forward dosimetry approach, the urinary levels of AAMA were directly compared with the non-cancer BEs and the BEs based on the human equivalent point of departure (BE_{POD}) derived for AA in urine and reported by Hays and Aylward (2008). Two complementary risk characterization methods were applied to interpret the obtained urinary levels of AAMA at GM and P95-levels in a risk context:

i) Prioritization schemes for direct comparison to the BEs and the BE_{PODs} which provide an initial estimation of whether the exposure to AA has low (below BE), medium (between BE and BE_{POD}), or high (above BE_{POD}) risk priority (Hays and Aylward, 2009)
ii) Calculation of the hazard quotient (HQ), doing the ratio between the observed AAMA levels in urine and the BE (Gurusankar et al., 2017). If the calculated HQ is lower than 1, it is considered that there is a low risk derived from AA exposure. At the same time, the hazard quotient derived from BE_{PODs} had been calculated too.

It is important to note that these cut-off values cannot be used to separate "safe" from "unsafe" exposures.

3. Results

3.1. Characteristics of the studied population and urinary levels of acrylamide metabolites

Finally, a total of 505 urine samples were included in the present study, since 35 of them had abnormal Cre concentrations. The education level of the majority was "university degree" (51.1%) and most of them were not smokers (74.3%). The mean (±SD) concentration of urinary Cre was 127 ± 6 mg dL^{-1} and the mean SG value was 1.020, ranging between 1.002 and 1.035.

Descriptive statistics for urinary AA metabolites levels corrected by volume, Cre and SG for the studied population are presented in Table 2. Urinary levels of AAMA, GAMA-3 and AAMA-Sul stratified by smoking status of the population, due to its high influence on them (see Table 3), are presented in Tables S1 and S2.

In all analysed urine samples, urinary metabolites of AA were quantified above Limit of Quantification (LoQ). The highest concentrations were observed for AAMA, which is considered the primary AA metabolite in urine, with a GM and 95th percentile (P95) of 84 and 418

Table 2
Descriptive statistics of the urinary AA metabolites (UAAM) concentrations from Spanish adult population (n = 505).

Metabolite	LoQ	DF (%)	Min.	P25	P50	AM	GM	P75	P95	Max.	SD	Units
AAMA	0.25	100	9	45	81	130	84	149	418	1071	147	$\mu g \cdot L^{-1}$
AAMA-Sul	0.25	100	3	15	26	39	26	43	129	265	38	
GAMA-3	0.25	100	1	6	12	17	11	22	52	140	17	
[a]∑UAAM	–	–	64	276	510	779	520	880	2430	5441	814	$nmol \cdot L^{-1}$
Ratio GAMA/AAMA	–	–	0.01	0.09	0.13	0.17	0.14	0.22	0.40	1.20	0.12	dimensionless
$AAMA_{Cre}$	–	–	12	43	68	102	74	121	307	885	96	$\mu g \cdot g\ Cre^{-1}$
$AAMA\text{-}Sul_{Cre}$	–	–	4	14	21	31	23	37	90	173	27	
$GAMA\text{-}3_{Cre}$	–	–	1	6	10	14	10	17	37	78	12	
[b]$\sum UAAM_{Cre}$	–	–	73	276	431	613	462	722	1788	4497	538	$nmol \cdot g\ Cre^{-1}$
$AAMA_{SG}$	–	–	15	60	98	152	106	174	455	1584	164	$\mu g \cdot L^{-1}$
$AAMA\text{-}Sul_{SG}$	–	–	5	19	30	46	33	51	142	499	46	
$GAMA\text{-}3_{SG}$	–	–	2	8	14	20	14	25	64	276	22	
[c]$\sum UAAM_{SG}$	–	–	90	383	598	916	662	1002	2621	9863	932	$nmol \cdot L^{-1}$

LoQ = Limit of quantification; DF (%) = Detection Frequency (% samples > LoQ); Min = Minimum; P = percentile; AM = Arithmetic mean; GM = geometric mean; Max = Maximum; SD = Standard deviation.
Suffix (Cre): Results of urinary AA metabolites corrected by urinary creatinine (Cre) levels.
Suffix (SG): Results of urinary AA metabolites corrected by specific gravity (SG) values.
[a] $\sum UAAM = AAMA + AAMA\text{-}Sul + GAMA\text{-}3$.
[b] $\sum UAAM_{Cre} = AAMA_{Cre} + AAMA\text{-}Sul_{Cre} + GAMA\text{-}3_{Cre}$.
[c] $\sum UAAM_{SG} = AAMA_{SG} + AAMA\text{-}Sul_{SG} + GAMA\text{-}3_{SG}$.

Table 3
Results of the linear regression model fitted for the log-transformed ∑UAAM, with its corresponding regression coefficients and 95% confidence interval.

	Variable	Standardized coefficients (β) (95% confidence interval)	p-value	R^2
∑UAAM	Intercept	−26.300 (−30.300–−22.271)	<0.001	0.499
	Specific gravity	28.356 (24.409–32.304)	<0.001	
	Smoking status: Smoker	0.385 (0.326–0.444)	<0.001	
	Smoking status: Passive smoker	0.015 (−0.060–0.090)	0.696	
	Potato fried products (kg in last 24 h)	0.178 (0.039–0.316)	0.012	
	Biscuits and pastries (kg in last 24 h)	0.389 (0.043–0.736)	0.028	

$\mu g\ L^{-1}$, respectively, whereas the GM and P95 values of AAMA-Sul and GAMA-3 were 26 and 129 $\mu g\ L^{-1}$, and 11 and 52 $\mu g\ L^{-1}$, respectively. Upon adjusting for Cre levels, the urinary levels of AA metabolites decreased. However, when adjusting for SG values, the levels increased, highlighting the impact of the adjustment on the observed levels. The mean value of the oxidation ratio (GAMA-3/AAMA) was 0.17, ranging from 0.01 to 1.20.

3.2. Statistical analysis

The Spearman's correlation matrix of three AA metabolites using the three correction methods is presented in Tables S3, S4 and S5. These table shows a high correlation among all metabolites (p-value <0.01), with correlation coefficients between 0.636 and 0.921, corroborating that AA is the common parent compound for the three analysed biomarkers in urine.

The results of the prediction model for the log-transformed ∑UAAM are depicted in Table 3. The obtained multiple linear regression model explained the 49.9% of the data variability. As it can be seen, SG values (28.356, $p < 0.001$) and the smoking status (0.385, $p < 0.001$) have a strong and positive influence on AA metabolite levels, which could explain a high dependence of AA biomarkers on this physiological value, and corroborating that tobacco smoke is one of the highest contributors of AA exposure. Furthermore, the amount of potato fried products (0.178, $p < 0.050$) and biscuits and pastries (0.389, $p < 0.050$) consumed in the last 24 h were also related to higher ∑UAAM levels.

3.3. EDIs and risk assessment

EDIs of AA were calculated for the entire analysed population based on urinary AA metabolites. Three correction methods (Cre, volume and SG) were employed, as shown in Fig. S1. The GM values ranged from 1.33 to 2.13 $\mu g\ AA \cdot kg\text{-}bw^{-1} \cdot day^{-1}$. Fig. 1 (for SG-adjusted values) and S2 (for Cre and volume-adjusted values) present the stratified EDIs based on the smoking status of the participants. Among smokers, the EDIs were two to three times higher (ranging from 2.52 to 4.19 $\mu g\ AA \cdot kg\text{-}bw^{-1} \cdot day^{-1}$) compared to non-smokers (1.07–1.66 $\mu g\ AA \cdot kg\text{-}bw^{-1} \cdot day^{-1}$) or passive smokers (1.04–1.80 $\mu g\ AA \cdot kg\text{-}bw^{-1} \cdot day^{-1}$). There was no significant difference between non-smokers and passive smokers ($p = 0.721$) (see Table S6). Furthermore, considering the impact of fried potato products or biscuits and pastries consumption within the last 24 h on urinary AA metabolite levels, the EDIs adjusted by SG were stratified accordingly (see Fig. S3). Approximately 40% of the study population consumed at least one of these foods within the last 24 h. Those who consumed these foods showed higher EDIs compared to individuals who did not consume them, as observed in the GM distribution level.

To assess these EDIs in a risk assessment context, we calculated the corresponding MOE values using formula (4) (see Fig. S4). Fig. 2 (for SG-adjusted values) and S5 (for Cre and volume-adjusted values) present MOEs stratified by smoking status of the participants. Fig. S6 showcases MOEs for SG-adjusted values stratified by the consumption of fried potato products or biscuits and pastries in last 24 h. In the population under study, MOE values at the 95th percentile distribution level were lower than 125 (see Figs. S4 and S6). About all of the subgroups studied

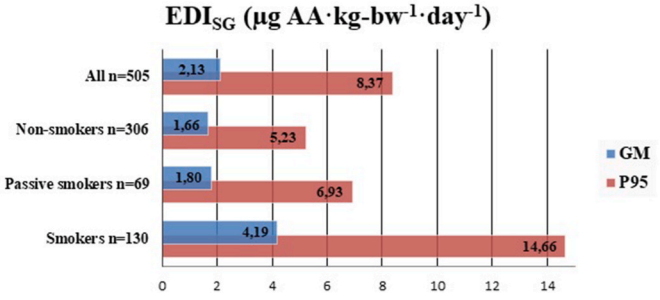

Fig. 1. Estimated daily intakes of AA calculated from ∑UAAM adjusted by specific gravity values and stratified by smoking status, at GM and P95 distribution levels.

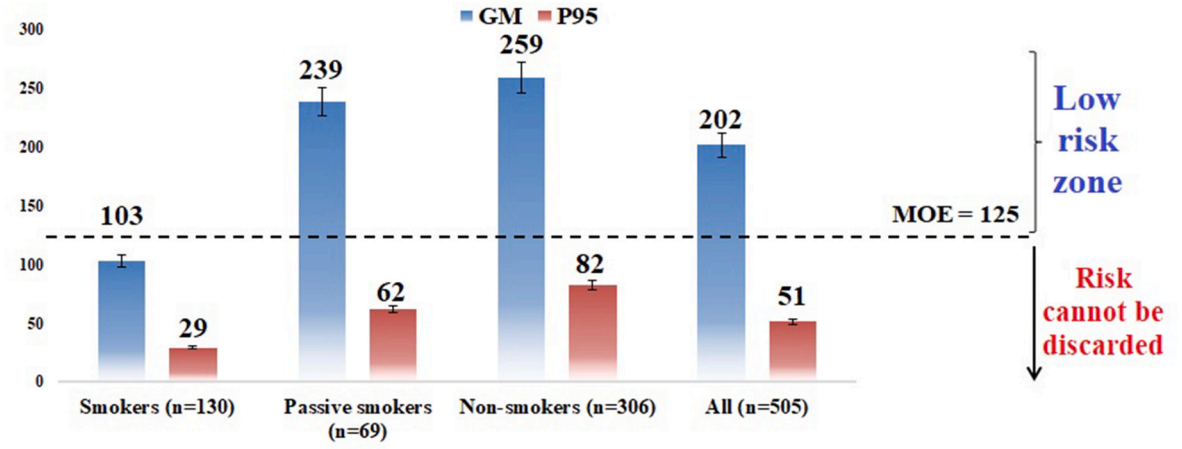

Fig. 2. Risk assessment reserve dosimetry approach: representation of Margin of Exposure (MOE) (dimensionless) at GM and P95 distribution levels adjusted by specific gravity values and stratified by smoking status.

here, only smokers had MOEs, adjusted by SG and by volume, below 125 at GM (see Fig. 2 and S5b).

Regarding the forward dosimetry strategy, urinary AAMA levels were directly compared with the corresponding BEs. The risk prioritization schemes and HQs for the studied population are presented in Fig. S7 and the same information stratified by smoking status is shown in Fig. 3 (for SG-adjusted values) and S8 (for Cre- and volume-adjusted values). The risk prioritization diagrams indicate that the mean levels of AAMA were 5–8 times higher than the BE, but lower the BE_{POD} (see Fig. S7). Therefore, exposure to AA in the Spanish adult population can be considered of medium risk priority. However, if the 95th percentile value is considered, the risk priority became high because AAMA levels exceeded the high-priority cut-off value.

Regarding the smoking status, the mean AAMA levels of passive- and non-smokers were quite similar (see Fig. 3a) and fell between the two reference values, resulting in HQs ranging from 3.5 to 6.7 and 3.7 to 6.3, respectively. In contrast, smokers had higher HQs compared to passive and non-smokers, as well as the entire population (9.2–16.9 vs 4.6 to 8.2). This is due to their elevated AAMA levels, which were 0.9–1.7 times higher than the BE_{POD} (see Fig. 3b, S9 and S10). In this case, the stratification by the consumption of fried potato products or biscuits and pastries in last 24 h is not reported, as it yielded similar results to the entire studied population.

In summary, the reverse dosimetry only indicated a concern due to "non-neoplastic effects" in the studied population at P95 and in smokers. In contrast, the forward dosimetry approach showed that AA exposure may be of public health concern for non-cancer risks in all population subgroups studied, as all calculated HQs were greater than 1.

4. Discussion

This is the first and representative AA biomonitoring study in the adult population of the Valencian Region of Spain. The three most relevant urinary AA metabolites, AAMA, GAMA-3, and AAMA-Sul, were quantified in the occupational adult population residing in the Valencian Region of Spain in order to assess risks of AA exposure. Due to the representativeness of the sample, this data may be used as reference values for this Spanish population group. The P50 of urinary AA levels was used to compare the obtained levels with previously reported HBM studies on adults (see Fig. 4) because many of them do not provide GM values. These studies, along with others, are described in more detail in Table S7.

In humans, the metabolic and toxicokinetic properties of AA indicate that AAMA is the predominant urinary metabolite (Kocadağli and Gökmen, 2016). As a result, it is the most analysed biomarker in HBM studies conducted through urine. This is in accordance with the results obtained here, since AAMA presented the highest levels followed by AAMA-Sul and GAMA-3. The high detection frequencies observed in the present study were similar to those reported by other authors (Mojska et al., 2016; Qian et al., 2021; F. Fernández et al., 2022a; Liao et al., 2022), indicating a widespread AA exposure among different nationalities. Spanish women (F. Fernández et al., 2022a) and Taiwanese adults (Liao et al., 2022) were observed to have similar AAMA levels to the participants of the BIOMOVAL project. However, adults from USA (CDC, 2021), China (Wang et al., 2020a, 2020b) and South Korea (Lee et al., 2019), as well as Polish women (Mojska et al., 2016), presented lower AAMA levels than those obtained in our research. On the other hand, women and children residing in the Valencian Region of Spain (F. Fernández et al., 2022a, 2022b) presented higher AAMA-Sul levels than the Spanish adult population evaluated here. Finally, GAMA-3 levels in the studied population were similar to those reported for women living in Spain (F. Fernández et al., 2022a) and Poland (Mojska et al., 2016), but higher than in the Chinese adult population (Wang et al., 2020a, 2020b).

As expected, smokers presented higher levels than passive- and non-smokers in the present study. Regarding the non-smoker population residing in the Valencian Region of Spain, women (F. Fernández et al., 2022a) and the adult population presented similar levels of urinary AA metabolites, but seemed to be more exposed to AA than non-smokers from China (Wang et al., 2020a, 2020b), Poland (women) (Mojska et al., 2016) and Italy (Frigerio et al., 2020). On the other hand, smokers from Poland (women) (Mojska et al., 2016) and USA (Keith et al., 2020) showed higher urinary AA metabolite levels than smokers of the BIOMOVAL project. Nevertheless, these levels were also higher than urinary AA metabolite levels observed in smokers from China (Wang et al., 2020a, 2020b) and Italy (Frigerio et al., 2020).

The Spanish adult population studied here showed a median ratio of GAMA-3/AAMA of 0.13. For comparison purposes, and considering that some HBM studies did not report this ratio, it was calculated using the median concentrations reported for GAMA-3 and AAMA in each study (see Table S7). As it can be observed, the Chinese adult population (Wang et al., 2020a, 2020b) had similar ratios to the Spanish population under study (0.15 vs 0.13). However, higher ratios were found in Polish (Mojska et al., 2016) and Spanish women (F. Fernández et al., 2022a), as well as in the U. S. adult population (Keith et al., 2020). According to smoking status, the ratio of GAMA-3/AAMA value for smokers was lower than those calculated for passive- and non-smokers in the present study (0.11 vs 0.14), which is in line with the results reported by Mojska et al. (2016), Wang et al. (2020a, 2020b) and F. Fernández et al. (2022a). Those variations among populations may be responsible for

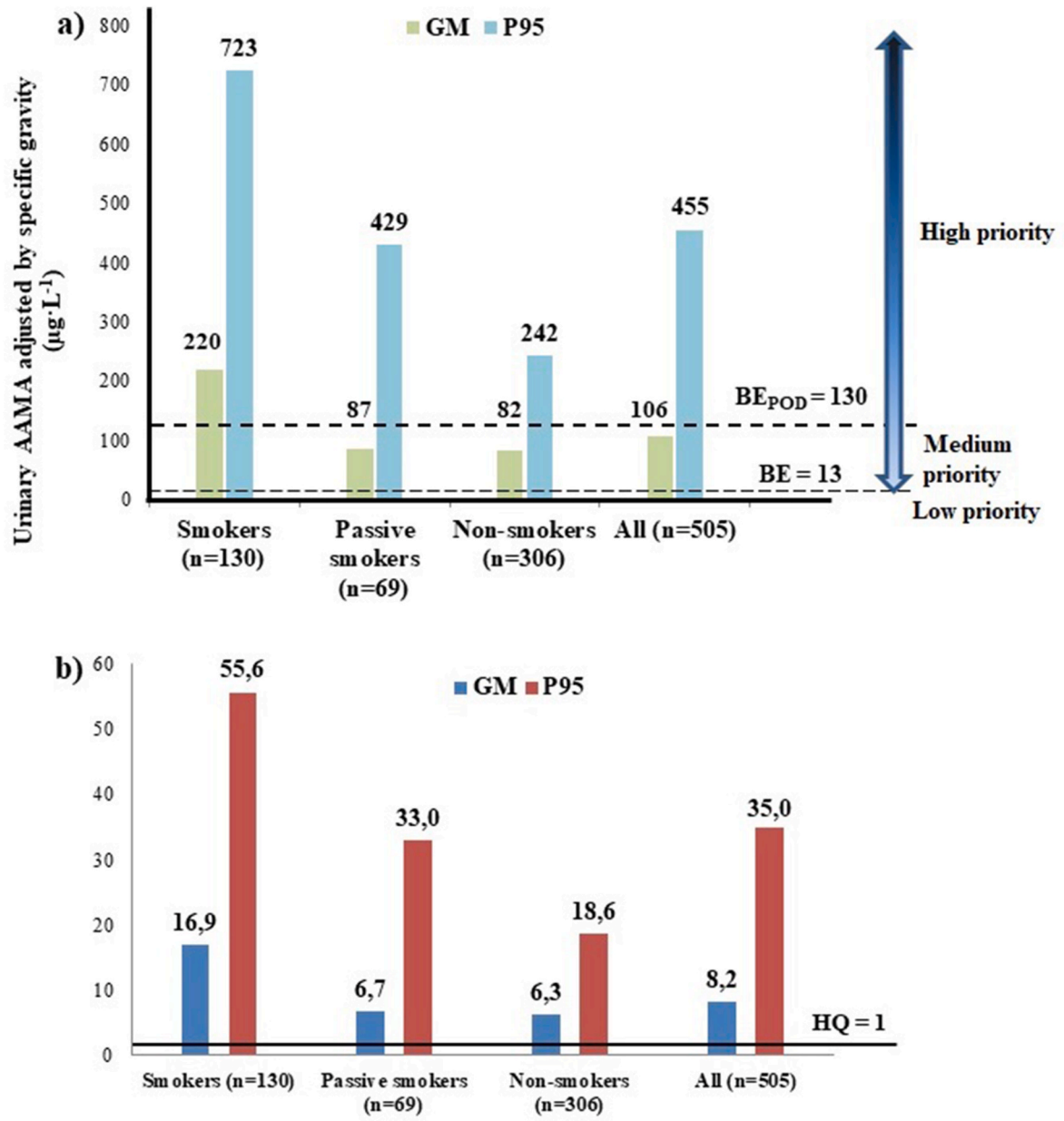

Fig. 3. a) Prioritization scheme representation and b) Hazard Quotients (HQs) (dimensionless) calculated using BEs, adjusted by specific gravity values and stratified by smoking status.

differences in the individual metabolism or the type of urine samples analysed in each study. On the other hand, the differences between smokers and passive- and non-smokers might be explained by a possible inhibition of the cytochrome P450 2E1 system by nicotine from smoke (Bjellaas et al., 2005).

The identification of potential predictors of human exposure to AA is crucial in order to develop more precise mitigation and control strategies. According to our results, and as highlighted in many recent studies (Mojska et al., 2016; Liao et al., 2022; F. Fernández et al., 2022a), smoking is highly related to metabolites levels of AA in urine (see Table 3). On the other hand, no significant differences between urinary AA metabolite levels in passive- and non-smokers were observed (see Table S6). Another strong influence factor was the specific gravity due to the dependence on the hydration state of the samples. Similar associations were found in other studies, but with the Cre levels (Mojska et al., 2016; F. Fernández et al., 2022a). Among the dietary habits of the population evaluated here, the amount of potato fried products or biscuits and pastries consumed in the last 24 h was observed to potentially increase urinary AA metabolite levels. Similar findings were also reported on Spanish children (F. Fernández et al., 2022b). On the other hand, although coffee is reported as a one of the main sources of dietary exposure to AA according to European authorities (EFSA, 2015), no association with urinary AA metabolite levels was observed in this study ($p > 0.05$). This is in line with Molina-Périz et al. (2016), who claimed that coffee consumption is responsible for only 3% of the daily intake of AA in the Spanish population. The association observed here could be explained by the similarities in coffee consumption in the Spanish population, as 70% of the BIOMOVAL participants drank a similar quantity of coffee in the last 24 h before sampling.

In this study, the calculated geometric mean EDIs of the entire population were in the range reported by EFSA (0.4–1.9 µg AA·kg-bw^{-1}·day^{-1}) (EFSA, 2015), except the EDI adjusted by SG (2.13 µg AA·kg-bw^{-1}·day^{-1}). These values were higher than the EDIs reported by Molina-Périz et al. (2016) for the adult population of Spain and the

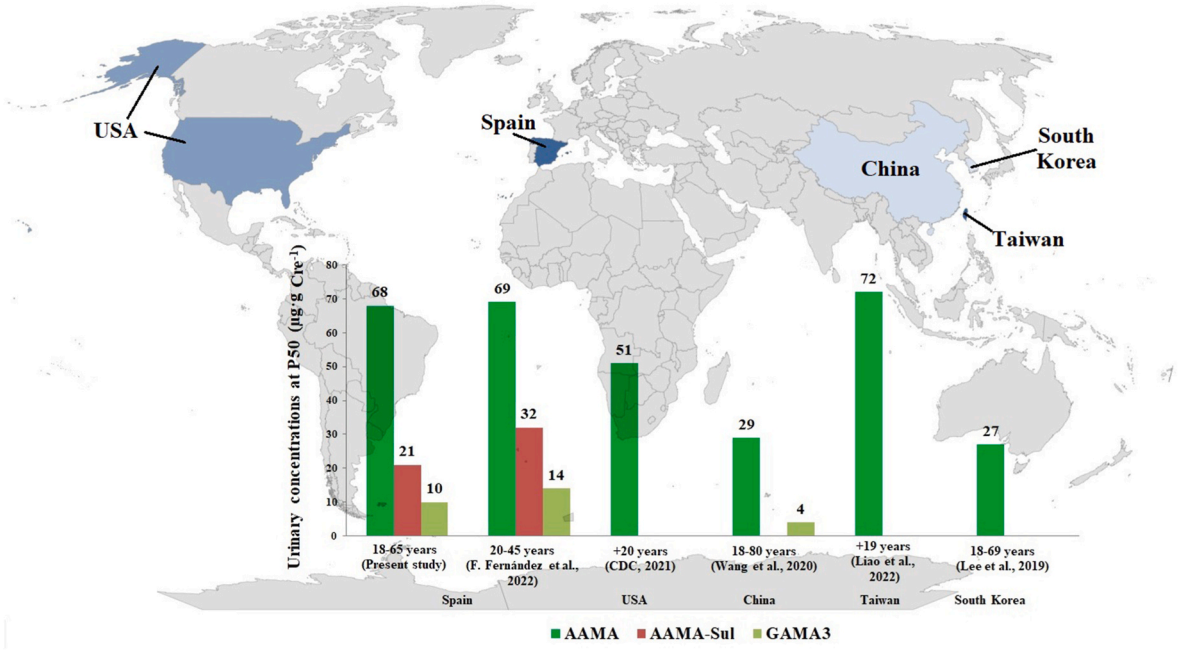

Fig. 4. Comparison of current HBM data of urinary AA metabolites in general population from different countries.

Valencian Region based on diet studies (0.75 and 0.54 μg AA·kg-bw^{-1}·day^{-1}, respectively). Furthermore, when considering the 95th percentile values, the EDIs exceeded the maximum upper limit reported by EFSA (3.4 μg AA·kg-bw^{-1}·day^{-1}) (EFSA, 2015). These differences could be explained because, in addition to diet, other sources of AA exposure such as tobacco smoke were considered. EDIs obtained in the present study were similar to the EDIs reported by F. Fernández et al. (2022a) in Spanish women, and both were higher than the EDIs obtained in other HBM studies in adults from Poland, Portugal and Taiwan (Mojska et al., 2016; Costa et al., 2022; Liao et al., 2022), where values ranged from 0.16 to 0.72 μg AA·kg-bw^{-1}·day^{-1}. The inclusion of urinary AAMA-Sul levels in the EDI calculations, which could lead to greater robustness of these estimations, could explain the differences observed. The calculated EDIs shown here highlight the high exposure to AA in the Spanish adult population, being the highest EDIs reported so far.

According to the results of the reverse dosimetry approach, the geometric mean MOEs obtained in the present study were similar to those observed in Spanish women and children (F. Fernández et al., 2022a, 2022b). However, these values were lower than MOE values reported in the European adult population (1075–717) (EFSA, 2015). The EFSA CONTAM Panel advised that current levels of dietary exposure to AA in adults are not of concern with respect to its non-neoplastic effects. Nevertheless, the MOEs obtained at P95 were far below 125, showing a potential health risk in the studied population. Regarding the smoking status, smokers showed the lowest MOEs, being considered as the highest exposed population group. Finally, risk assessment based on forward dosimetry showed that AAMA concentrations were higher than the BE in all cases, with HQ values higher than 1, indicating that AA exposure should be considered as a medium-risk priority in the Spanish adult population, and evidencing the need for more mitigation and prevention measures in public health.

5. Strengths and limitations of the study

The questionnaire employed in the BIOMOVAL project was accurately designed to reflect AA exposure in the target population with specific questions related to it. In addition, the representativeness of the sample size together with the high detection frequencies obtained for the three main urinary AA metabolites, allows obtaining results of a high statistical power and deriving reference values of internal AA exposure for the adult population living in the Valencian Region. However, the questionnaire was self-reported and only included occupational population due to the recruitment conditions.

6. Conclusions

In the present study, urinary levels of the three main AA metabolites in the Spanish adult population were presented for the first time. AAMA, GAMA-3 and AAMA-Sul were quantified in all analysed samples with GM of 84, 11 and 26 μg L^{-1}, respectively. The calculated EDIs of AA based on its urinary metabolites ranged between 1.33 and 2.13 μg AA·kg-bw^{-1}·day^{-1} at GM. Tobacco smoke and the amount of potato fried products and, biscuits and pastries consumed in last 24 h were identified as potential predictors of exposure to AA.

The risk assessment estimations provided evidence on the wide exposure to AA in the target population, which was observed to pose a potential public health concern. For this reason, it is necessary to increase the number of public health mitigation and prevention measures to minimize exposure to AA among citizens, as well as to continuously monitor the exposure of the population to this compound in subsequent HBM studies.

Author statement

Borja Peris: Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization, Software, Formal analysis; Olga Pardo: Conceptualization, Writing – review & editing, Supervision; Sandra F. Fernández: Methodology, Writing – review & editing; Pablo Dualde: Writing – review & editing; Clara Coscollà;: Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Borja Peris Camarasa reports financial support was provided by Government of Valencia.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.121896.

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