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Chemometric Study of the Correlation between Human Exposure to Benzene and PAHs and Urinary Excretion of Oxidative Stress Biomarkers

Flavia Buonaurio [1], Enrico Paci [2], Daniela Pigini [2], Federico Marini [1], Lisa Bauleo [3], Carla Ancona [3] and Giovanna Tranfo [2,*]

1 Department of Analytical Chemistry, Sapienza University, Piazzale Aldo Moro, 5, 00185 Rome, Italy; flavia.buonaurio@uniroma1.it (F.B.); federico.marini@uniroma1.it (F.M.)
2 INAIL Research, Department of Occupational and Environmental Medicine, Epidemiology and Hygiene, Via di Fontana Candida 1, 00078 Monte Porzio Catone (RM), Italy; e.paci@inail.it (E.P.); d.pigini@inail.it (D.P.)
3 Department of Epidemiology Lazio Regional Health Service, Via Cristoforo Colombo, 112, 00154 Rome, Italy; l.bauleo@deplazio.it (L.B.); c.ancona@deplazio.it (C.A.)
* Correspondence: g.tranfo@inail.it

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Abstract: Urban air contains benzene and polycyclic aromatic hydrocarbons (PAHs) which have carcinogenic properties. The objective of this paper is to study the correlation of exposure biomarkers with biomarkers of nucleic acid oxidation also considering smoking. In 322 subjects, seven urinary dose biomarkers were analyzed for benzene, pyrene, nitropyrene, benzo[a]pyrene, and naphthalene exposure, and four effect biomarkers for nucleic acid and protein oxidative stress. Chemometrics was applied in order to investigate the existence of a synergistic effect for the exposure to the mixture and the contribution of active smoking. There is a significant difference between nicotine, benzene and PAH exposure biomarker concentrations of smokers and non-smokers, but the difference is not statistically significant for oxidative stress biomarkers. The PAH biomarkers are those which best correlate with all the oxidative stress biomarkers. Results suggest that 8-Oxo-7,8-dihydroguanine and protein nitro-oxidation 3-nitrotyrosine are the most sensitive biomarkers for the exposure to the urban pollutant mixtures and that a synergic effect of the mixtures exists. All the oxidative stress biomarkers studied drive the increase in the oxidative stress biomarkers in the subjects having higher exposures. Chemometrics proved to be a powerful method for the interpretation of human biomonitoring data.

Keywords: human biomonitoring; inhalation exposure; urban pollution; central Italy; oxidative stress; smoking; chemometrics

1. Introduction

Urban air pollution represents a problem for human health. According to the World Health Organization (WHO), 98% of cities in low- and middle-income countries with more than 100,000 inhabitants do not meet WHO air quality guidelines, nor do 56% of cities in high-income countries [1]. The term air pollution can refer to very different exposure mixtures, but, among the major air pollutants, benzene and polycyclic aromatic hydrocarbons (PAHs) are listed, present both in the gas and in the particulate phase [2]. Benzene is a IARC (Intenational Agency for Research on cancer) class 1 carcinogenic substance. It is a ubiquitous pollutant of indoor and outdoor air, as even though in Europe its use is restricted by the European regulation on Registration and Evaluation of Chemicals (REACH), it is still present in industrial processes, combustion of organic and natural gases and motor fuels, as a 1% volume of benzene is allowed in gasoline. Benzene is also an important

component of cigarette smoke, which significantly contributes to direct and indirect human exposure. The Directive 2008/50/EC on ambient air quality and cleaner air for Europe sets objectives for ambient air quality in order to protect human health and the environment as a whole; these objectives relate to sulfur dioxide, nitrogen dioxide, particulate matter, lead, benzene, and carbon monoxide. For benzene, the limit value has been set at 5 $\mu g/m^3$ as the annual average since 1 January 2010.

Polycyclic aromatic hydrocarbons (PAHs) have been considered in the Directive (EU) 2019/130 of The European Parliament and of The Council of 16 January 2019 (amending Directive 2004/37/EC on the protection of workers from the risks related to exposure to carcinogens or mutagens at work), which declared that certain polycyclic aromatic hydrocarbon mixtures, particularly those containing benzo[a]pyrene, meet the criteria for classification as carcinogenic, category 1. Exposure to such mixtures may occur during work involving burning processes, such as from combustion engine exhausts and high temperature combustion processes, among others. The possibility of significant uptake of these mixtures through the skin was also identified. They are ubiquitous substances produced by the incomplete combustion of organic matter, such as in traffic emissions, and heating and power sources, such as oil, coal, and biomass in outdoor areas, and cooking, residential heating, and tobacco smoke in indoor areas.

Human biomonitoring of specific biomarkers is useful for determining human exposure to, and metabolism of, potentially toxic and carcinogenic components of air pollution, and distinguishing exposures due to air pollution from those resulting from tobacco smoke. The following validated chemically specific biomarkers are considered the most suitable for studies of air pollution and cancer: urinary 1-hydroxypyrene, phenanthrene metabolites, S-phenyl mercapturic acid, urinary or blood Cd, 8-hydroxydeoxyguanosine, and F2-isoprostanes such as 8-iso-PGF2α. This suite of biomarkers will reliably establish exposure to carcinogenic polycyclic aromatic hydrocarbons, benzene and Cd, and will also provide critical information on oxidative damage and inflammation, both of which are important in carcinogenesis [3].

The present study analyzed a suite of selected exposure biomarkers, namely S-phenylmercapturic acid (SPMA) and t,t-muconic acid (t,t-MA) for benzene, 1-hydroxypyrene (1-OHPy) for pyrene, 6-hydroxynytropyrene (6-OHNPy) for Nitropyrene, 3-hydroxybenzo[a] pyrene (3-OHBaP) for benzo[a]pyrene, 1-hydroxynaphthalene (1-OHNAP) and 2-hydroxynaphthalene (2-OHNAP) for naphthalene, cotinine for cigarette smoking, a group of three urinary biomarkers of nucleic acid oxidation and one of protein nitro-oxidation: 8-Oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), 8-Oxo-7,8-dihydroguanosine (8-oxoGuo), 8-Oxo-7,8-dihydroguanine (8-oxoGua), and 3-nitrotyrosine (3-NO_2Tyr). The biomarkers were determined in the urine samples of 426 subjects participating in the so called "ABC" study (ABC is the Italian acronym for Ambiente e Biomonitoraggio Civitavecchia) [4]. The ABC biomonitoring study consisted of the collection of urine samples between May 2013 and December 2014 from a population randomly selected from municipality registers of the city of Civitavecchia (Italy). The studied group included 1016 subjects aged 35–69 years, without occupational exposure to PAHs.

In the present study, chemometric methods are applied to the biomonitoring results in order to investigate more deeply the effect of the inhalation exposure to the mixture of benzene and PAHs present in urban air on oxidative damage of nucleic acids and tyrosine, the existence of an additive or even synergistic effect, and the contribution of smoking.

2. Experiments

2.1. Study Population

The present study is a part of the larger "ABC" study in which urinary samples were collected between May 2013 and December 2014 on a population randomly selected from municipality registers of the city of Civitavecchia (Italy); the study protocol was approved by the local ethics committee.

The studied group included 1016 subjects aged 35–69 years, non-occupationally exposed to benzene and to polycyclic aromatic hydrocarbons, out of which results for two subgroups are reported.

For the first subgroup of 426 subjects (126 smokers and 300 non-smokers), the urinary concentrations of 8-oxoGua, 8-oxoGuo, 8-oxodGuo, SPMA, Cotinine, 1-OHPy, 6-OHNPy, 3-OHBaP, 1-OHNAP, and 2-OHNAP were determined. For the second subgroup of 322 subjects (102 smokers and 220 non-smokers), the urinary concentration of 3-NO_2 tyrosine was also added to the same set of biomarkers.

Each subject was asked to complete a questionnaire for collecting information on age, lifestyle and food habits, smoking, drug use, working activities, hobbies, and use of chemical products.

Before providing the urine sample, all subjects gave written informed consent for participation in the study.

2.2. Urine Sample Collection

Urine samples were collected in sterile plastic containers. In the laboratory, they were aliquoted into three 15 mL screw-cap tubes, and were immediately frozen at −20 °C until analysis. The final concentration of the analytes was expressed in micrograms per gram (μg/g) of creatinine to normalize values with respect to urine dilution variability. Urinary creatinine was determined by the method of Jaffè using an alkaline picrate test with UV/Vis detection at 490 nm [5]. The samples with a creatinine concentration higher than 3 g/L or lower than 0.3 g/L were discarded and the corresponding volunteers were excluded from the study in accordance with the American Conference of Governmental Industrial Hygienists' (ACGIH) recommendation [6]. The smoking status was assessed using the urinary concentration of cotinine and the cut-off value for the definition of smoker was set at urinary cotinine ≥ 100 μg/g of creatinine [7].

2.3. Chemicals and Supplies

The analytical reference standards of 1- and 2-OHNaP, 1-OHPy, 6-OHNPy 3-OHBaPy, 8-oxoGua, 8-oxodGuo, and 8-oxoGuo were purchased by Spectra 2000 s.r.l. (Spectra 2000, Rome, Italy). The isotope labelled internal standards 2-OHNAP d_7, 1-OHPy d_9, 3-OHBaPy d_{11}, DL-SPMA-3,3 d_2, ($^{13}C^{15}N_2$) 8-oxodGuo, and ($^{13}C^{15}N_2$) 8-oxoGuo were obtained from CDN Isotopes Inc. (Pointe-Claire, QC, Canada). ($^{13}C^{15}N_2$) 8-oxoGua (98%) was obtained from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA), 3-NO_2Tyr was purchased by Cayman Chemical Company (Ann Arbor, MI, USA), and 3-NO_2Tyr d3 from TRC (Toronto, ON, Canada). Glacial acetic acid 30% NH_3, dimethyl sulfoxide, sodium hydroxide solution (50–52% in water, CHROMASOLV® gradient grade 99.9% methanol and acetonitrile for HPLC/MS, and 99.9% carbon disulfide with low benzene content were obtained from Sigma Aldrich (Saint Louis, MO, USA). Purified water was obtained from a Milli-Q Plus system (Millipore Milford, MA, USA). The solid phase extraction (SPE) cartridges, Sep-Pak Plus C18 (10 mL, 500 mg) were supplied by Waters (Waters S.p.A., MI, Italy) and Strata Screen-X (55 μm, 70 Å) 500 mg/6 mL cartridges by Phenomenex (Phenomenex, Torrance, CA, USA). An Anotop 10LC syringe filter device (0.2 μm pore size, 10 mm diameter) was purchased from Whatman Inc. (Maidstone, UK). A Sinergi Fusion C18 column (150 × 4.6 mm, 4 μm), Luna® 5 μm C8 100 Å, LC column 250 × 4.6 mm, and Kinetex Polar C18 column 100 A (150 × 4.6 mm, 2.6 m) (Phenomenex, Torrance, CA, USA) were used throughout the study.

2.4. Analysis of Urine Samples

All the urine samples were analyzed on a Series 200 LC quaternary pump (PerkinElmer, Norwalk, CT, USA) coupled with an AB/Sciex API 4000 triple-quadrupole mass spectrometry detector equipped with a Turbo Ion Spray (TIS) probe. The instrument was calibrated using polypropylene glycol, and the resolution was adjusted to a peak full width at half maximum (FWHM) of 0.7 Th over the range of *m*/*z* 100–1000. Detection was in the multiple reaction monitoring (MRM) mode, and parameters were optimized for the analytes by the automated "infusion quantitative optimization"

procedure and subsequently refined by flow injection analysis (FIA) using the pure standards. DL S-phenylmercapturic acid (DL-SPMA), cotinine, and the deuterium-labelled internal standards DL-SPMA-3,3-d_2 and cotinine-d_3 were determined following the method previously used and validated in our laboratory [8]. The precursor→product ionic transitions monitored were in the negative ion mode: 238.1→109.1 for SPMA, and 240.1→109.1 for SPMAd_2 and in the positive ion mode 177.3→80.10 for cotinine, and 180.3→80.10 for cotinine-d_3. Monohydroxylated PAHs (OH-PAHs), urinary metabolites of PAHs, were set up and validated in our laboratory for five urinary metabolites: 1-hydroxypyrene (1-OHPy), 1-hydroxynaphthalene (1-OHNAP), 2-hydroxynaphthalene (2-OHNAP), 3-hydroxybenzo[a] pyrene (3-OHBaPy), and 6-hydroxynytropyrene (6-OHNPy) [9]. The precursor→product ionic transitions monitored were in the negative ion mode: 217.1→189.1 for 1-OHPy and 226.0→198.1 for 1-OHPy-d_9, 262.0→231.9 for 6-OHNPy, 267.1→239 for 3-OHBaPy and 278.0→250.0 for 3-OHBaPy-d_{11}, 142.9→115.2 for 1 and 2-OHNAP, and 149.8→122 for 2-OHNAP-d7. Limits of detection were as follows: 1-OHPy = 0.00040 μg/L; 6-OHNPy = 0.00030 μg/L; 3-OHBaPy = 0.000070 μg/L; 1-OHNAP = 0.0033 μg/L; 2-OHNAP = 0.015 μg/L. Data lower than limit of detection (LOD) were substituted with LOD/2.

The concentrations of 8-oxoGua, 8-oxoGuo, 8-oxodGuo, and 3-NO_2tyrosine were determined by following the method described by [10] with some modifications such as mode of thawing of the sample, dilution solvents, chromatographic column, and mobile phases and introduction of dosage 3-NO_2tyrosine and its internal standard. Before the analysis, samples were thawed in lukewarm water at about 37 °C, vortexed, and centrifuged at 10,000× *g* for 5 min; the urine supernatant was added of internal standard and was injected into the high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) system. The precursor/product ionic transitions monitored (positive ion mode) were 168.0→140.0 and 171.0→143.0 for 8-oxoGua and its internal standard (($^{13}C^{15}N_2$) 8-oxoGua), 284.3→168.0 and 287.13→171.1 for 8-oxodGuo and its internal standard (($^{13}C^{15}N_2$) 8-oxodGuo), 300.24→168.2 and 303.24→171.0 for 8-oxoGuo and its internal standard (($^{13}C^{15}N_2$) 8-oxoGuo), 226.99→181.0, and 229.99→184.0 for 3-NO_2tyrosine and its internal standard (3-NO_2tyrosine d_3), respectively. The 1.5 version of Analyst® software (AB Sciex. Framingham, MA, USA) was employed for instrument control.

2.5. Statistical Analysis

Statistical analyses were performed using Analysis ToolPak, an add-in program for Microsoft Office Excel, after checking the log-normality of the distribution of data. Parametric methods (Pearson correlation, *t* test for independent variables) were then used on the log-transformed data. In all tests, a *p*-value lower than 0.05 (two-tailed) was considered as statistically significant.

In order to understand the effect of the exposure to the mixture of benzene and PAHs (and smoking in smokers) on oxidative damage of nucleic acids and tyrosine, chemometric methods were used to further process the complete data from 322 subjects. In particular, to explore the relationship between the two blocks of data (exposure and oxidative stress biomarkers), a multiblock approach based on the ComDim algorithm was exploited. Multiblock techniques are data processing approaches which start from the consideration that more than a single set of data is available to describe the samples under investigation, and they have the advantage of preserving the information about the nature of the individual matrices during the modeling stage. Within this framework, the ComDim approach belongs to the family of unsupervised techniques, which are aimed at exploratory data analysis: exploratory analysis focuses on summarizing the main characteristics of a dataset—i.e., identifying the relations between samples and/or variables, often through tools which allow a straightforward graphical display. ComDim (also known as Common components and specific weight analysis, CCSWA [11]) is a multiblock exploratory technique which aims at extracting so-called common components from multiple data matrices sharing one dimension in common (usually the same set of samples). Components are extracted so as to account for as much joint variability among the data matrices as possible and ranked according to decreasing amount of total explained variance. Projection

of the analyzed samples along these common components provides new coordinates (scores) which can be plotted to highlight possible similarities or dissimilarities among individuals. For the sake of interpretation, the sample scores along the common components are used to calculate individual sets of loading vectors for each of the analyzed data matrices. Inspection of the loadings allows interpretations to be made in chemical terms of the sample distribution patterns observed in the score plot. The contribution of each data block to the definition of a common component is expressed by a quantity called salience. Further information can be found in the original paper [11].

In a second stage of the study, the possibility of quantitatively relating the two sets of markers through a multivariate regression model was also explored. To this purpose, multiple linear regression (MLR) [12] was adopted. MLR can be considered as the direct generalization of univariate least squares to the case where more than one independent variable is measured. In particular, in the case where a single response y is modeled as a function of a set of independent variables $x_1, x_2, \cdots, x_p$, a direct proportionality is assumed through the definition of the regression coefficients b_i which constitute the model parameters:

$$y = b_0 + b_1x_1 + b_2x_2 + \cdots + b_px_p$$

with p being the number of independent variables. Model building corresponds to finding the optimal values of the regression coefficients given the data and the optimal set of parameters is found according to the least squares principle—i.e., it is the one minimizing the sum of squared residuals between the true values of the responses and the model predictions.

In the present study, all the chemometric models have been implemented by means of function written in-house and running under the MATLAB (v. 8.6 release R2015b; The Mathworks, Natick, MA, USA) environment.

3. Results and Discussion

3.1. Descriptive Statistics

Table 1 lists the characteristics of the 426 subjects studied in terms of smoking habit, gender, and body mass index (BMI).

Table 1. Characteristics of subjects.

Parameter	Sex		Total
	Male	Female	
All subjects	188 (44.1%)	238 (55.9%)	426
Smokers	46	80	126
Non-smokers	142	158	300
BMI (kg/m^2)-median	28.01	25.23	26.47

The concentration levels of 10 analytes for all subjects of different subgroups have been reported in Table 2 in terms of mean with the standard deviation, 5th, 50th and 95th percentiles.

The detection rate of urinary PAHs is usually low in the general population/non-smokers without occupational exposure. In this case, the percentage of data below the respective LOD was 31 for 1-OHPy, 74 for6-OHNPy, 85 for 3-OHBaP, and 23 for 1-OHNAP and 2-OHNAP.

Subjects were classified into smokers and non-smokers according to their urinary concentration of cotinine. We defined smoker subjects as having a urinary cotinine concentration ≥ 100 μg/g of creatinine [7].

Table 2. Urinary concentrations of 10 biomarkers in subgroup of 426 subjects, in smokers and non-smokers.

All Subjects n. 426	**μg/g Creatinine**										
	8-oxoGua	**8-oxoGuo**	**8-oxodGuo**	**SPMA**	**Cotinine**	**1-OHPy**	**6-OHNPy**	**3-OHBaP**	**1-OHNAP**	**2-OHNAP**	**1 + 2 OHNAP**
Mean (STD)	55.21 (38.19)	9.70 (5.55)	5.01 (3.99)	0.75 (1.50)	807.29 (2262.8)	0.06 (0.13)	0.06 (0.28)	4.91×10^{-3} (0.02)	3.44 (6.14)	3.18 (4.93)	6.62 (9.16)
5th	13.80	2.64	0.45	3.45×10^{-3}	1.87	9.69×10^{-5}	5.85×10^{-5}	1.32×10^{-5}	9.03×10^{-4}	3.64×10^{-3}	0.01
50th	45.37	8.86	4.40	0.17	11.38	0.01	1.70×10^{-4}	3.30×10^{-5}	1.36	1.44	3.47
95th	126.88	20.11	11.98	3.50	3847.49	0.32	0.42	0.04	15.25	11.55	25.41
Smokers n. 126	**8-oxoGua**	**8-oxoGuo**	**8-oxodGuo**	**SPMA**	**Cotinine**	**1-OHPy**	**6-OHNPy**	**3-OHBaP**	**1-OHNAP**	**2-OHNAP**	**1 + 2 OHNAP**
Mean (STD)	55.16 (39.51)	9.21 (5.38)	4.84 (3.54)	2.11 (2.21)	2702.59 (3502.4)	0.12 (0.17)	0.07 (0.24)	0.01 (0.02)	6.73 (7.36)	5.61 (6.31)	12.33 (11.16)
5th	16.48	2.39	0.31	0.13	287.91	8.05×10^{-5}	4.99×10^{-5}	1.16×10^{-5}	9.83×10^{-4}	3.25×10^{-3}	0.01
50th	44.67	8.27	4.60	1.49	1359.88	0.06	1.41×10^{-4}	2.61×10^{-5}	4.15	4.05	10.15
95th	131.88	19.14	10.99	6.07	9529.08	0.44	0.64	0.03	22.77	14.24	32.44
Non-smokers n. 300	**8-oxoGua**	**8-oxoGuo**	**8-oxodGuo**	**SPMA**	**Cotinine**	**1-OHPy**	**6-OHNPy**	**3-OHBaP**	**1-OHNAP**	**2-OHNAP**	**1 + 2 OHNAP**
Mean (STD)	55.23 (37.69)	9.91 (5.62)	5.08 (4.17)	0.17 (0.23)	11.27 (13.09)	0.04 (0.10)	0.05 (0.29)	4.61E-03 (0.02)	4.94 (3.94)	2.80 (3.80)	4.22 (6.91)
5th	12.63	2.87	0.48	3.07×10^{-3}	1.58	1.01×10^{-4}	6.63×10^{-5}	1.50×10^{-5}	8.96×10^{-4}	3.93×10^{-3}	0.02
50th	45.87	9.01	4.31	0.11	5.93	0.01	1.84×10^{-4}	3.57×10^{-5}	0.94	0.95	2.61
95th	124.96	20.14	12.63	0.69	39.75	0.17	0.09	0.04	6.24	8.13	12.07

The concentration levels of 3-NO2Tyr for the subgroup of 322 subjects, 102 Smokers, and 220 non-smokers have been reported in Table 3 in terms of mean with the standard deviation, 5th, 50th and 95th percentiles.

Table 3. Urinary concentrations of 3-NO_2Tyr in the subgroup of 322 subjects, in smokers and non-smokers expressed in µg/g creatinine.

	All Subjects n. 322	**Smokers n. 102**	**Non-Smokers n. 220**
Mean (STD)	8.07 (4.15)	8.24 (4.67)	7.98 (3.90)
5th perc.	3.01	3.01	3.02
50th perc.	7.34	7.38	7.29
95th perc.	15.73	17.24	14.80

A previous study involved 446 of the 1016 ABC study volunteers. In this case, the urine samples were analyzed to determine the concentration of benzene and nicotine metabolites—SPMA, t,t-MA, and cotinine and the three biomarkers of nucleic acid oxidative stress (8-oxoGua, 8-oxoGuo, and 8-oxodGuo) on a subsample of 131 subjects. With reference to nucleic acid oxidation, there were no significant differences in the subgroup for the three biomarkers' concentrations in the urine between smokers and non- or ex-smokers, while for smokers there was a significant correlation of urinary 8-oxodGuo with SPMA and cotinine, indicating that an effect of smoking on nucleic acid oxidation exists, even if it is probably masked by other factors [8].

In this study, the difference in the mean urinary concentrations of the biomarkers between 102 smokers and 220 non-smokers has been explored by means of the T-test on their log-transformed values.

There is no statistically significant difference between the nucleic acid oxidative stress biomarker concentrations of smokers and non-smokers, nor for 3-NO_2Tyr. On the contrary, the difference is highly statistically significant ($p << 0.01$, both one-tailed and two-tailed) not only for cotinine and SPMA, but also for the PAH biomarkers 1-OHPy, 1-OHNAP, and 2-OHNAP, confirming what was found in the previous study, carried out using a smaller number of subjects [8].

The difference between the mean values of biomarkers in males and females was also explored by means of the T-test on their log-transformed values, and results are reported in Table 4, together with the standard deviations.

All the biomarkers' concentrations in females are statistically significantly higher than in males, except for 8-oxodGuo. This difference between genders is usually found in most biomonitoring studies.

The Pearson correlations among all the tested biomarkers concentrations have also been studied regarding their log-transformed values on the complete data from the subgroup of 322 subjects, and results are reported in Table 5.

Table 4. Mean urinary concentrations of 11 biomarkers in males and females expressed in μg/g Creatinine.

	8-oxoGua	8-oxoGuo	8-oxodGuo	SPMA	Cotinine	1-OHPy	6-OHNPy	3-OHBaP	1-OHNAP	2-OHNAP	1 + 2 OHNAP	$3NO_2Tyr$
Females						**n. 238**						**n. 181**
Mean	62.63 **	10.27 *	5.30	0.96 **	1104.28 **	0.08 **	0.07 *	0.01 *	4.51 **	3.96 *	8.46 **	8.90 **
STD	42.39	6.05	4.51	1.73	2826.79	0.15	0.33	0.02	7.53	5.8	10.99	4.53
Males						**n. 188**						**n. 141**
Mean	46.00	8.99	4.65	0.47	431.27	0.04	0.04	0.004	2.09	2.210	4.29	6.99
STD	29.63	4.78	3.20	1.10	1120.51	0.09	0.19	0.02	3.25	3.32	5.29	3.32

Difference female vs. males * $p < 0.05$; ** $p < 0.01$.

Table 5. Pearson's correlation on the log-transformed values of oxidative stress biomarkers and exposure biomarkers on 322 complete results. (Underlined: $p < 0.05$; bold: $p < 0.01$).

	Age	BMI	8oxoGua	8oxoGuo	8oxodGuo	$3NO_2Tyr$	Cotinine	SPMA	1OHPy	6OHNPy	3OHBaP	1OHNAP	2OHNAP
Sex	**0.154**												
BMI	**0.201**												
8oxoGua	**0.255**	−0.010											
8oxoGuo	**0.145**	−0.003	**0.335**										
8oxodGuo	0.077	−0.009	**0.212**	**0.840**									
$3NO_2Tyr$	**0.160**	−0.019	**0.633**	**0.623**	**0.493**								
Cotinine	−0.084	−0.038	**0.138**	−0.001	0.035	0.134							
SPMA	−0.049	−0.027	0.060	−0.069	−0.076	0.085	**0.638**						
1OHPy	0.010	0.057	**0.283**	**0.223**	**0.227**	**0.258**	**0.344**	0.069					
6OHNPy	0.122	0.022	**0.306**	**0.257**	**0.258**	**0.328**	0.117	−0.053	**0.441**				
3OHBaP	0.040	0.030	−0.168	0.112	0.224	−0.256	0.059	−0.020	**0.286**	0.275			
1OHNAP	−0.086	−0.058	0.061	−0.147	−0.114	−0.020	**0.453**	**0.428**	0.108	−0.011	−0.115		
2OHNAP	−0.119	0.034	0.015	0.040	0.120	0.073	**0.402**	**0.232**	**0.303**	**0.262**	0.159	**0.240**	
1 + 2 OHNAP	−0.088	0.047	0.105	−0.013	0.019	0.039	**0.371**	**0.270**	**0.241**	**0.181**	0.069	**0.848**	**0.622**

The interpretation of the *r* values is expressed by the following: $0.1 < |r| < 0.3$ small/weak correlation; $0.3 < |r| < 0.5$ medium/moderate correlation; $0.5 < |r| \ldots$ large/strong correlation. Two different levels of significance were considered, so that underlined values correspond to $p < 0.05$, whereas values marked in boldface showed $p < 0.01$.

The nucleic acid oxidative stress biomarkers are all positively correlated among themselves and with 3-NO_2Tyr, while the 8-oxoGua is also correlated to the subjects' ages [13]. The benzene biomarker SPMA is strongly correlated to the urinary cotinine concentration, according to the fact that smoking is the main source of benzene exposure in smokers, but it is weakly correlated to the oxidative stress biomarkers if the smoker group is considered. This result is not in accordance with our previous study, where a stronger association was found in smokers, but in this study the number of subjects is higher (126 smokers vs. 31). In non-smokers, no association was found in both studies, as the benzene exposure is very low, rendering the exploration of its effects very difficult.

The PAH biomarkers 1-OHPy, 6-OHNPy, and 3-OHBAP are the biomarkers that best correlate with all the oxidative stress biomarkers. This result is in accordance with the fact that PAHs are a class of carcinogenic agents—in particular, benzo[a]pyrene is a IARC class 1 carcinogen [14]. Because 1- and 2-OHNAP are both metabolites of naphtalene, the sum of the two is also considered. It looks as though 2-OHNAP is more related to oxidative stress than 1-OHNAP. It could depend on the metabolic pathway, but we have no explanation for this, nor was there any literature on it.

As part of the ABC biomonitoring study, the same urine samples were previously analyzed in order to determine the concentrations of the five PAHs metabolites. Results have shown a correlation between exposure to PAHs and smoking; in particular, urinary excretion of 1- and 2-OHNAP and 1-OHPy is influenced by cigarette smoking, while in the case of 3-OHBaP and 6-OHNPy, smoking habit is only partially responsible for their excretion [15].

3.2. Chemometrics

In order to investigate deeper the effect of the inhalation exposure to the mixture of benzene and PAHs present in urban air on oxidative damage of nucleic acids and tyrosine, the existence of a synergistic effect, and the contribution of smoking, a strategy based on the use of multivariate chemometric methods was applied to the data of the 322 subjects, for which all the 12 variables were determined.

The distribution of each variable was examined and found to be log-normal for both the eight exposure and the four oxidative stress biomarkers. For the urinary cotinine concentration, the existence of two populations is evidenced, confirming the cutoff of 100 µg/g creatinine (Figure 1).

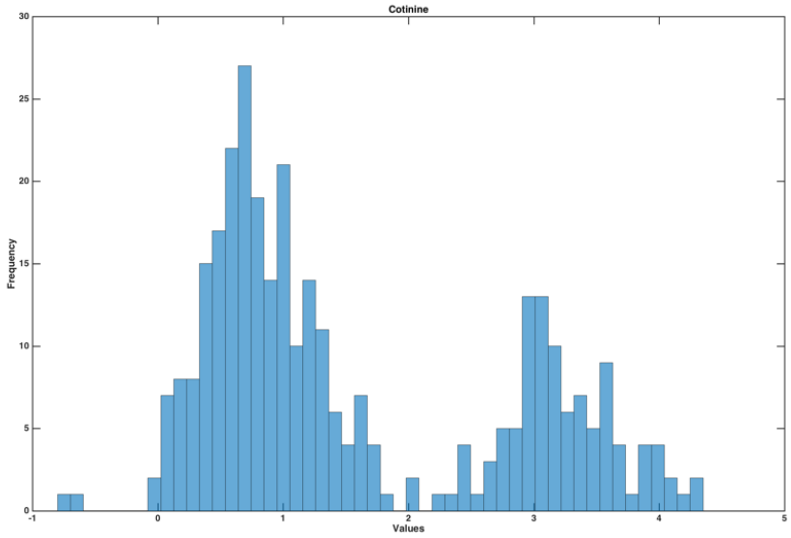

Figure 1. Distribution of $\log_{10}$-concentration values of urinary cotinine in 322 subjects.

A ComDim multiblock model was then calculated in order to explore the relations between the exposure biomarkers and the oxidative stress biomarkers. In particular, the largest part of the variance in the two blocks (more than 90%) was accounted for by the first two common components, whose scores and loadings are graphically displayed in Figure 2. The scores loadings were analyzed and are reported in Figure 2.

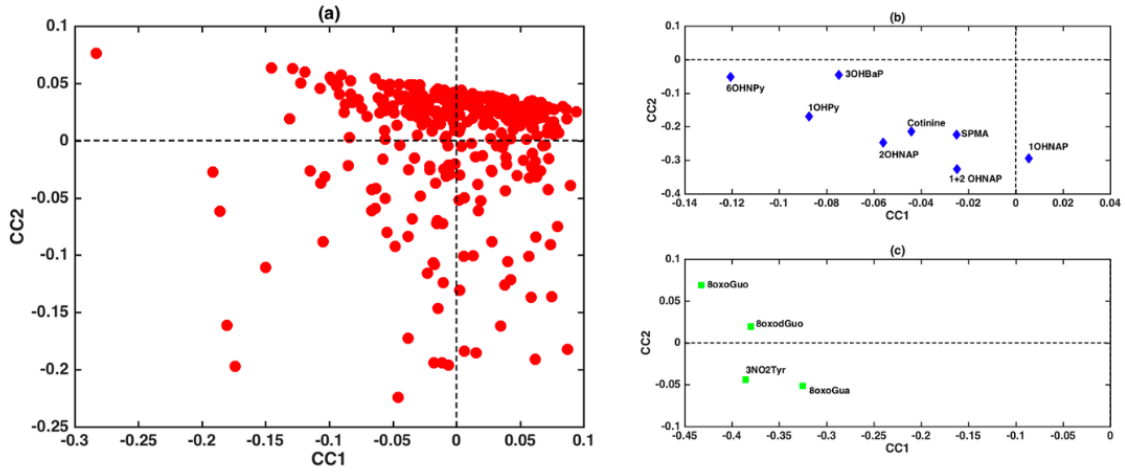

Figure 2. Results of ComDim modeling. (**a**) Projection of the samples (scores) on the first two common components (CCs) of the model; the variable loadings associated to the same common components are displayed in panels (**b**) and (**c**) for exposure and oxidative stress biomarkers, respectively.

From this analysis, we can say that subjects having higher levels of exposure to the considered pollutants, and therefore higher levels of the corresponding urinary exposure biomarkers, also present higher levels of two of the four oxidative stress effect biomarkers, namely 8-oxoGua and 3-NO_2Tyr. Therefore, these two biomarkers seem to be the more sensitive to the mixture of urban pollutants.

The multiblock analysis was also applied to other characteristics of the studied subjects, gathered from the purposely administered questionnaire: sex, age, body mass index (BMI), and the smoking habit quantified by means of the urinary cotinine concentration, setting a cutoff of 100 µg/g creatinine.

Concerning sex, there is not a significant difference in the distribution of data between males and females, but the highest values belong to female subjects, confirming what is shown in Table 4.

No differences were also evidenced in the distribution for BMI and age, while for smokers and non-smokers there is a clear separation between the results of the two groups, confirming that smokers have a high level of exposure to benzene and PAHs and higher levels of 8-oxoGua and 3-NO_2Tyr (see Figure 3).

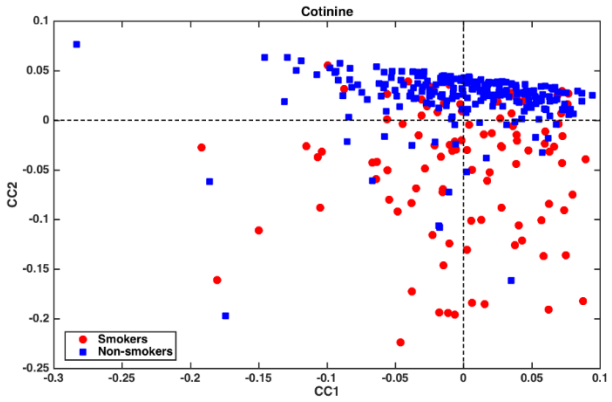

Figure 3. Score plot resulting from the ComDim analysis (same as Figure 2a), colored according to smoker/non-smoker status defined based on urinary cotinine levels.

A multivariate regression analysis was also carried out on the same dataset, in order to predict the different biomarkers of effect on the basis of the eight exposure biomarkers, which are produced by the exposure to the mixture of benzene and PAHs.

Considering all the 322 subjects, the multivariate regression does not evidence any particular tendency. This can be justified by the fact that, as the subjects volunteers form a general population group, no particular exposure sources are involved, and the oxidative stress biomarkers are also influenced by other variables not considered in this study, such as the general health status, use of drugs or medication, exposure to pollutants such as pesticides or to other dangerous substances used for hobbies, etc.

However, if the regression analysis is restricted to the 47 subjects with the highest level of exposure (corresponding to the points in the lower left corner of the scores plot in Figure 2a; data reported as Table S1 in the Supplemental Materials), a better correlation can be observed. In particular, the cross-validated predictions (eight cancelation groups) of MLR models relating the individual oxidative stress biomarkers to the set of exposure biomarkers are displayed in Figure 4. The *r* values defining the correlation between the predicted and the measured values are 0.60 for 8-oxoGua, 0.57 for 8-oxodGuo, 0.46 for 8-oxoGuo, and 0.52 for the 3-NO_2Tyr, indicating that in these subjects the exposure to the considered pollutants is correlated to an increase in the oxidative stress biomarkers.

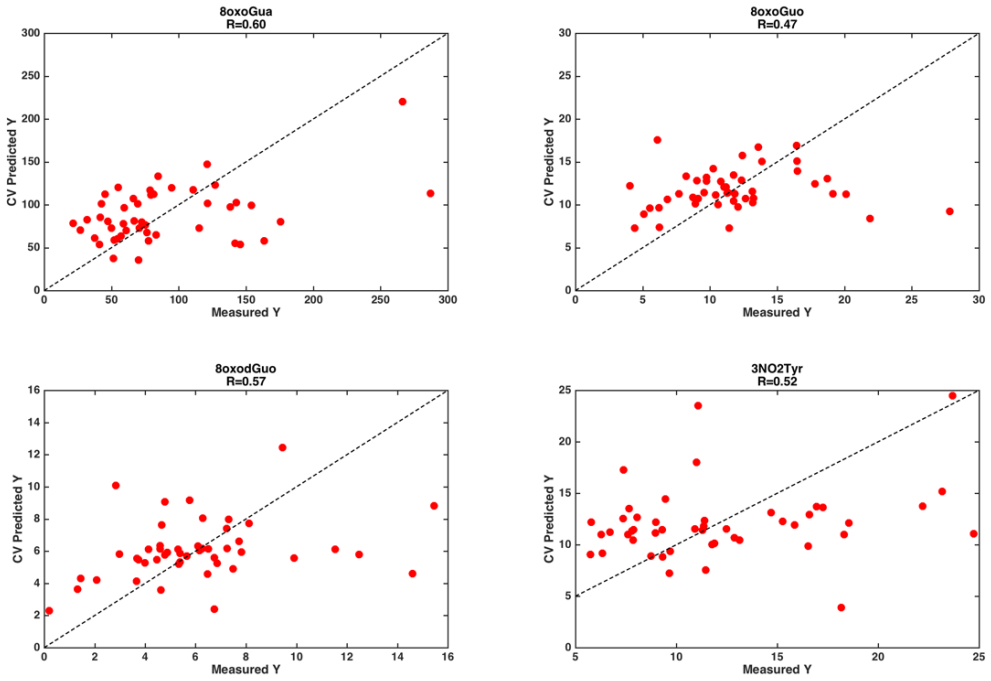

Figure 4. Results of multiple linear regression (MLR) modeling between individual oxidative stress biomarkers and the set of exposure biomarkers for the selected subset of 47 individuals with the highest level of exposure: plots of cross-validated predictions vs. observed values.

From an interpretation standpoint, inspection of the regression coefficients of the calibration models built on the reduced set of 47 individuals allows the identification of the exposure metabolites mostly related to each of the oxidation markers. In the case of 8-oxoGua and 8-oxoGuo, it was found that only 1-OHNAP, 2-OHNAP, and 1-2OHNAP significantly contributed to the regression model, the latter being negatively correlated to the response, whereas the others have positive coefficients. On the other hand, almost all the exposure markers (with the only exception of 2OHNAP) were found to significantly contribute to the model for the prediction of 8-oxodGuo: sign of the coefficients was positive for most of the exposure markers, with the exception of SPMA and 10HPy. Lastly, all exposure markers were found to significantly contribute to the model for the prediction of 3-NO_2Tyr;

in this case, 1-OHNAP, 2-OHNAP, and 1-2OHNAP showed a negative coefficient, suggesting they are anti-correlated to the response, while all the other substances had positive values.

On the other hand, if univariate regression models are fitted between any of the exposure biomarkers and the individual markers for oxidative stress, the quality of the predictions and, in particular, the corresponding correlation coefficients, are lower than the ones obtained from the multivariate models, indicating the existence of an additive effect of the mixture compared to the single pollutant effect.

4. Conclusions

Results of this study combine the results of biomonitoring 322 subjects from a general population group of Central Italy for 11 different biomarkers. They show that the benzene biomarker SPMA is strongly correlated to the urinary cotinine concentration, according to the fact that smoking is the main source of benzene exposure in smokers, but it does not seem to be strongly correlated to the oxidative stress biomarkers when data are explored using a traditional statistical technique (linear bivariate correlation). The PAH biomarkers 1-OHPy, 6-OHNPy, and 3-OHBAP are the biomarkers that best correlate with all the oxidative stress biomarkers. This result is in accordance with the fact that PAHs are a class of carcinogenic agents; in particular, benzo[a]pyrene is an IARC class 1 carcinogen.

However, the deepest insight into the data is provided by chemometric analysis: from this elaboration, we can say that subjects with the highest levels of exposure to the considered pollutants, and therefore the highest levels of the corresponding urinary exposure biomarkers, also present the highest levels for two of the four oxidative stress effect biomarkers, namely 8-oxoGua and 3-NO_2Tyr. Moreover, the multiblock analysis showed that for smokers and non-smokers there is a clear separation between the results of the two groups, confirming that smokers have a high level of exposure to benzene and PAHs and higher levels of 8-oxoGua and 3-NO_2Tyr. Therefore, 8-oxoGua and 3-NO_2Tyr seem to be the most sensitive biomarkers to the mixture of urban pollutants.

In conclusion, as the subjects are a group of volunteers from the general population, for which no particular exposure sources are involved, the oxidative stress biomarkers studied are influenced also by variables not considered in this study, such as general health status, use of drugs or medication, exposure to pollutants such as pesticides or to other dangerous substances used for hobbies etc.

However, in the subjects with higher exposure biomarkers levels, there is a very good correlation between the exposure and the effect biomarkers indicating that, in these subjects, the exposure to the considered pollutants is driving the increase in the oxidative stress biomarkers. Additionally, the existence of an additive effect of the mixture compared to the single pollutant effect is showed by the results of chemometric analysis, which proved to be a powerful method for the interpretation of human biomonitoring data.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4433/11/12/1341/s1, Table S1: Biomarkers levels in the 47 subjects with the highest level of exposure.

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