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**ABSTRACT**

**Background:** Processed meat intake is associated with a higher risk of colorectal and stomach cancers, coronary artery disease, and type 2 diabetes and with higher mortality, but the estimation of intake of different processed meat products in this heterogeneous food group in epidemiological studies remains challenging.

**Objective:** This work aimed at identifying novel biomarkers for processed meat intake using metabolomics.

**Methods:** An untargeted, multi-tiered metabolomics approach based on LC-MS was applied to 33 meat products digested in vitro and secondly to urine and plasma samples from a randomized crossover dietary intervention in which 12 volunteers consumed successively 3 processed meat products (bacon, salami, and hot dog) and 2 other foods used as controls, over 3 consecutive days. The putative biomarkers were then measured in urine from 474 subjects from the European Prospective Investigation into Cancer and Nutrition (EPIC) cross-sectional study for which detailed 24-h dietary recalls and FFQs were available.

**Results:** Syringol and 4 derivatives of syringol were found to be characteristic of in vitro digests of smoked meat products. The same compounds present as sulfate esters in urine increased at 2 and 12 h after consumption of smoked meat products (hot dog, bacon) in the intervention study. The same syringol sulfates were also positively associated with recent or habitual consumption of smoked meat products in urine samples from participants of the EPIC cross-sectional study. These compounds showed good discriminative ability for smoked meat intake with receiver operator characteristic areas under the curve ranging from 0.78 to 0.86 and 0.74 to 0.79 for short-term and habitual intake, respectively.

**Conclusions:** Four novel syringol sulfates were identified as potential biomarkers of smoked meat intake and may be used to improve assessment of smoked meat intake in epidemiological studies. This trial was registered at clinicaltrials.gov as NCT03354130. *Am J Clin Nutr* 2019;110:1424–1433.

**Keywords:** smoked meat, processed meat, dietary biomarkers, metabolomics, syringol, syringol sulfate

## Introduction

A large body of epidemiological and preclinical evidence shows a role for processed meat consumption in colorectal and stomach cancer etiology and the consumption of processed meat has been classified as carcinogenic to humans by the International Agency for Research on Cancer (IARC) (1). Processed meat intake has also been associated with a higher risk of overall mortality (2), coronary artery disease, and type 2 diabetes (3). Processed meat refers to all meat that has been processed by salting, curing, smoking, etc. to enhance flavor or preservation. As a food group, processed meat is very heterogeneous and can be categorized by processing method and ingredients into fresh processed meat, cured meat pieces, raw-cooked products, precooked-cooked products, raw (dry) fermented sausages, and dried meat (4). There is considerable regional variability in processed meat consumption, with high consumption in northeastern Europe and Central America and low consumption in Africa and most parts of Asia (5). The proportion of processed meat contributing to total meat intake varies between regions. Whereas processed meat contributes more than half of the energy intake from the food group "meat" (including fresh and processed meat) in some European countries


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such as Germany and Norway, its consumption contributes to <20% of the energy intake from this food group in countries such as Greece (6).

Several mechanisms have been proposed to explain the carcinogenicity of processed meat consumption (7), including the endogenous and exogenous formation of mutagenic *N*-nitroso-compounds (NOCs) from nitrate and nitrite used for curing, the induction of cell toxicity and proliferation by heme iron–triggered lipid oxidation, and the formation of genotoxic heterocyclic amines and polycyclic aromatic hydrocarbons (PAHs) during heating and smoking of meat.

Epidemiological evidence on the carcinogenicity of processed meat largely relies on the use of self-reported questionnaires to assess its intake. However, questionnaires are prone to inaccuracies owing to the recall biases of subjects (8) and often lack sufficient detail (e.g., information on food processing) to estimate the intake of different processed meat products. The use of biomarkers could improve the assessment of exposure to specific processed meat products and could be informative for understanding biological mechanisms.

To identify novel biomarkers for processed meat products, a multi-tiered metabolomic approach was successively applied to processed meat in vitro digests, to biospecimens from a dietary intervention study, and finally to biospecimens in the European Prospective Investigation into Cancer and Nutrition (EPIC) cross-sectional study. We focus in this article on the identification of novel biomarkers for smoked meat products.

## Methods

### Materials

Sulfur trioxide–pyridine complex was purchased from Alfa Aesar. All other chemicals and reagents were ordered from Sigma-Aldrich. Syringol sulfate, 4-methylsyringol sulfate, and 4-allylsyringol sulfate were synthesized from the commercially available syringol precursors as previously described (9): each syringol precursor (0.15 mmol) dissolved in pyridine (0.5 mL) with 0.15 mmol of pyridine:$SO_3$-complex added was heated at 60°C for 2 h. The reaction was quenched with water and acetonitrile was added before analysis.

### Meat in vitro digestion

Different brands of 4 fresh and 6 processed meat products and tofu were bought from local butchers and supermarkets in Lyon, France, and stored immediately at −20°C. All products were thawed at room temperature and products that are usually consumed heated were fried in a nonstick pan without added fat or oil at medium heat for 5 min on each side. All products were cut into pieces smaller than 1 cm and minced into a chunky homogenate in a blender. Aliquots of 1.5 g each were stored at −20°C. All samples were processed in triplicate. The in vitro digestion was carried out following the protocol described by Minekus et al. (10). Briefly, in the oral phase, 1.5 g homogenate was incubated with 1.5 mL buffer at 37°C for 2 min (no amylase was used in the oral phase because the products contain only small amounts of carbohydrate that were not of interest in this study). In the gastric phase, 3 mL of a buffer containing pepsin was added, the pH was adjusted to 3, and the resulting mixture was shaken for 2 h at 37°C. For the intestinal phase, 6 mL of a buffer containing bile extract and pancreatin was added and the pH was then adjusted to 7. The resulting mixture was shaken for 2 h at 37°C. The final mixture was centrifuged (15,000 × *g*, 15 min, 22°C) and the supernatant stored at −80°C until analysis.

### Randomized crossover dietary intervention study

Twelve nonsmoking healthy adults (6 men, 6 women) who were not using regular medication (mean ± SD BMI: 22.4 ± 2.6 kg/m$^2$, mean ± SD age: 31 ± 5.2 y) were recruited among IARC employees for a randomized crossover dietary intervention study (NCT03354130). A detailed FFQ that had been validated for assessing processed meat consumption (11) was completed by the volunteers before the study. Each volunteer consumed in a randomized order 5 different intervention diets containing either pork (135 g, fried), salami (67 g), hot dogs/frankfurter sausage (107 g, heated), bacon (104 g, fried), or tofu (178 g) for dinner on day 1 and lunch and dinner on days 2 and 3 (**Supplemental Table 1**). The amount of meat or tofu per meal was adjusted to contain 250 kcal. The volunteers all received the same amounts of meat and tofu. The additional food items of all meals during the test periods were provided ad libitum to the volunteers (Supplemental Table 1). Adherence to the intervention diet and the amount consumed were monitored by a food diary that was completed by the volunteers starting the day before, and continuing until the last day of the intervention period. A washout period of ≥10 d during which there was no diet restriction separated the 5 intervention periods. Volunteers were asked not to consume meat or fish on the day before the first intervention meal. Urine spot samples were collected 2 and 12 h (first morning void) after the first intervention dinner (day 1) of each intervention period in sterile urine beakers (BRAND®). A cumulative 12-h urine sample was collected after the last intervention dinner (day 3) starting

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Where authors are identified as personnel of the International Agency for Research on Cancer/WHO, the authors alone are responsible for the views expressed in this article and they do not necessarily represent the decisions, policy, or views of the International Agency for Research on Cancer/WHO.

Supplemental Tables 1–7 and Supplemental Figures 1–17 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

Data described in the article, code book, and analytic code will be made available upon request.

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Abbreviations used: EPIC, European Prospective Investigation into Cancer and Nutrition; IARC, International Agency for Research on Cancer; NOC, *N*-nitroso-compound; PAH, polycyclic aromatic hydrocarbon; ROC, receiver operating characteristic; 24HDR, 24-h dietary recall.

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**TABLE 1** Characteristics of the subjects participating in the cross-sectional study stratified by country[1]

| | All countries | Germany | Italy | France | Greece |
|---|---|---|---|---|---|
| *n* | 474 | 178 | 174 | 66 | 56 |
| Sex, *n* (female), *n* (male) | 279, 195 | 86, 92 | 98, 76 | 66, 0 | 29, 27 |
| Age, y | 53.9 ± 8.5 | 53.0 ± 8.8 | 54.1 ± 7.5 | 52.7 ± 7.0 | 58.0 ± 10.9 |
| BMI, kg/m$^2$ | 26.1 ± 4.3 | 26.2 ± 4.4 | 25.8 ± 3.8 | 23.4 ± 3.5 | 29.6 ± 3.8 |
| Short-term meat intake | | | | | |
| Total meat, g/d | 109.7 ± 98.64 | 122.3 ± 98.8 | 108.1 ± 105.1 | 107.7 ± 80.6 | 76.6 ± 90.1 |
| Smoked meat, g/d | 10.1 ± 32.5 | 25.4 ± 48.9 | 0.51 ± 4.04 | 3.1 ± 10.5 | 0 |
| Consumers of smoked meat, % | 16.9 | 38.8 | 1.72 | 12.1 | 0 |
| Habitual meat intake | | | | | |
| Total meat, g/d | 105.7 ± 54.8 | 107.3 ± 53.1 | 101.2 ± 50.0 | 138.0 ± 66.7 | 76.8 ± 38.3 |
| Smoked meat, g/d | 8.6 ± 15.1 | 22.2 ± 17.5 | 0.19 ± 1.40 | 1.6 ± 1.40 | 0 |
| Consumers of smoked meat, % | 40.7 | 94.4 | 2.87 | 30.3 | 0 |
| Short-term smoked fish intake, g/d | 1.6 ± 12.3 | 2.1 ± 16.1 | 0.57 ± 7.60 | 1.6 ± 7.50 | 3.3 ± 14.2 |
| Consumers of smoked fish, % | 3.2 | 3.4 | 0.57 | 6.1 | 7.1 |

[1] Values are mean ± SD for continuous variables and *n* for categorical variables.

from the first void after dinner and including the first morning void on day 4 in sterile urine collection containers (Urisafe®). During sample collection, containers were kept in a refrigerator. Upon entering the laboratory, urine containers were kept on ice. A fasting blood sample was taken by a certified nurse in the morning of the last intervention day (day 4) of each period (**Supplemental Figure 1**).

Urine samples were collected in sterile containers and kept refrigerated until arrival in the laboratory. They were then centrifuged (10 min, 3220 × *g*, 4°C) and the supernatant was stored at −80°C until analysis. The blood samples were taken in EDTA-containing vacutainer tubes and kept on ice until centrifugation (10 min, 2192 × *g*, 4°C). Plasma samples were divided into aliquots and stored at −80°C until analysis. The study was approved by the IARC Ethics Committee (IEC Project 17-12). All participants signed an informed consent form before their participation and procedures were carried out according to the principles expressed in the Declaration of Helsinki.

### EPIC cross-sectional study

Validation of smoked meat biomarkers was carried out in a subsample of the EPIC cohort, one of the largest prospective cohorts with >500,000 men and women from 10 European countries (12). Samples included in this study ($n = 474$) are a subset from the EPIC calibration study ($n = 36{,}994$) for which the 24-h dietary recall (24HDR) and urine sample were collected on the same day between 1995 and 1999 and the urine sample was stored at −20°C (**Supplemental Figure 2**). Participants also provided blood samples and FFQs with information about their habitual diet. The blood samples were not analyzed and therefore not included in this analysis. Characteristics of the study population can be found in **Table 1**. The ethical review boards from the IARC and from all local centers approved the study. All participants signed an informed consent form before their participation in the study.

The food intake data of the participants of the cross-sectional study were provided by the regional centers of the EPIC study. The intake of meat and fish that were described as smoked in the questionnaires or those which are usually smoked in their production (**Supplemental Table 2**) was calculated for every participant for both the 24HDR and FFQ and for meat and fish separately. For the 24HDR data, participants were grouped into nonconsumers (no smoked meat consumption in 24HDR), low-consumers (consumers below the median intake of all consumers), or high consumers (consumers above the median intake of all consumers). For the FFQ data, the distribution of consumption for all participants was taken into account to create intake categories. Participants that consumed <2 g smoked meat/d on average were classified as nonconsumers. The remaining participants were grouped into low consumers or high consumers if their intake was below or above the median of all consumers, respectively.

### LC-MS analyses

Meat in vitro digests and urine and plasma samples were analyzed by LC-MS. In vitro digest supernatants (30 μL) were mixed with cold acetonitrile (200 μL) and centrifuged (3220 × *g*, 10 min, 4°C). The supernatant was filtered on 0.2 $\mu$m Captiva ND plates (Agilent Technologies) and filtrates diluted 10 times with acetonitrile. Commercial liquid smoke (liquid smoke hickory and liquid smoke hard wood, Hot Danas GmbH; 10 μL) was diluted in 9:1 acetonitrile:water (vol:vol) (10 mL), centrifuged (1260 × *g*, 5 min, 22°C), filtered, diluted 100 times with the same solvent, and stored at −80°C until analysis. Urine samples were diluted with ultrapure water to the lowest specific gravity of any urine sample in the experiment to normalize their concentrations (13), centrifuged (2000 × *g*, 10 min, 4°C), and an aliquot of the supernatant was diluted 1:1 (intervention study) and 1:0.25 (cross-sectional study) with acetonitrile and stored at −80°C until analysis. Plasma samples (50 μL) were mixed with acetonitrile (300 μL), shaken for 2 min, centrifuged (2000 × *g*, 10 min, 4°C), and the supernatant was filtered and stored at −80°C. The samples were thawed at room temperature and dried by evaporation at 4°C overnight. The residue was reconstituted in 70 μL of a mixture of water with 5% acetonitrile.

Samples were then analyzed by LC-MS on an Agilent 1290 Binary LC system coupled to an Agilent 6550 quadrupole time-of-flight mass spectrometer with jet steam electrospray ionization source (Agilent Technologies), as previously described (14).

All samples of each study were randomized in a single batch (≤560 injections) and a quality control sample consisting of a pool of all samples in 1 batch was analyzed for every 8 study samples injected. Sample extracts [2 μL for all extracts with the exception of plasma samples from the intervention study (6 μL)] were injected onto a reverse-phase C18 column (Acquity UPLC HSS T3 2.1 × 100 mm, 1.8 $\mu$m, Waters) maintained at 45°C. A linear gradient made of ultrapure water and LC-MS grade methanol, both containing 0.05% (vol:vol) formic acid, was used for elution. The mass spectrometer was operated successively for each batch in positive and negative ionization mode, detecting ions across a mass range of 50–1000 daltons.

Annotation of MS features was done by first searching for metabolites with matching exact mass in publicly available databases like the Human Metabolome Database or METLIN as $[M + H]^+$, $[M + Na]^+$ (positive mode), or $[M-H]^-$ ions (negative mode) with a mass tolerance of 8 ppm (15, 16). Targeted fragmentation (MS/MS) spectra were acquired at 10, 20, and 40 eV. MS/MS spectra and retention time were compared to those of commercially available standards or in-house synthesized standards. Levels of confidence in the annotations were defined as proposed by Sumner et al. (17). Level 1 corresponding to a confirmed structure was based on full match of retention time and MS/MS spectrum with those of an authentic chemical standard. For level 2, no standard was available, and probable identification was based on similar physicochemical properties, isotope patterns, and MS/MS spectra. Annotation evidence is given in **Supplemental Figures 3–10**.

### Metabolomics data preprocessing

Raw data from each batch were processed individually. MassHunter software (Mass Profiler Professional version B.14.9.1, Qualitative Analysis version B06.00, and DA Reprocessor version B.05.00; Agilent Technologies) was used to process raw data in 2 steps. First, molecular features were extracted and aligned over all samples of the batch by mass (±15 ppm) and retention time (±0.1 min). All features that were present in ≥3 samples of the whole batch were used as a target in a second, recursive targeted feature search to create a feature table for each data set. Results of this run were aligned with the same criteria and signal areas were used for the subsequent statistical analysis. Missing values were imputed with 1 unless stated differently. Intensities for the intervention study plasma samples and the cross-sectional study samples were extracted from the raw data with the Profinder software (Agilent, version B.08.00), using a targeted feature extraction based on the following formula: mass tolerance ± 10 ppm, retention time ± 0.05 min. Chromatograms were inspected and peak areas for targeted MS features were corrected manually if necessary. Missing values from targeted feature extraction were imputed with the lowest intensity measured for each compound. Feature intensity data were log2 transformed for statistical analysis. MS feature data from in vitro–digested foods and data from intervention study urine samples were filtered by detection frequency; features that had missing data in >3 samples in every processed meat group or diet (e.g., hot dogs or bacon) were excluded.

### Statistical analysis

For the in vitro meat digests, an unpaired Welch 2-sample *t* test was conducted to identify all features that had different intensities in samples of processed meat products ($n = 36$) compared with nonprocessed meat products ($n = 63$) (adjusted $P$ value $< 0.05$ after correction for multiple testing using the Benjamini–Hochberg method with the false discovery rate set to 0.05). In subsequent analysis the same test was conducted to identify all features that had different intensities in samples of smoked meat products ($n = 27$) compared with nonsmoked meat products ($n = 72$).

The analysis of the in vitro digests suggests that smoke markers show the highest intensity in hot dog samples. A paired Student's *t* test was therefore used to identify features of the intervention study that were significantly different in urine samples collected after the hot dog diet compared with samples from the fried pork diet (adjusted $P$ value $< 0.05$ after correction for multiple testing using the Benjamini–Hochberg method with the false discovery rate set to 0.05). A paired Student's *t* test was used to assess if syringol sulfate compounds were significantly more elevated in plasma after hot dog intake than in plasma after fried pork intake.

For the analysis of the EPIC cross-sectional study, a Welch 2-sample *t* test was used to assess if urinary concentrations of syringol sulfate compounds were different between nonconsumers and high consumers of smoked meat according to 24HDR data. For sensitivity analysis, the same test was performed for subgroups by smoking status. To test for other factors that might correlate with intake of smoked meat, logistic regression models were built containing the biomarker intensities in urine, personal data of the participant (study center, smoking status, BMI, age, sex), and habitual intake of the major food groups as covariates and consumption/nonconsumption (as indicated by the questionnaire) as the outcome. To test if addition of the biomarker to the model increased the discriminatory power of the model, a 5-fold cross-validated receiver operating characteristic (ROC) analysis was performed by splitting the data into training sets (80% of samples) and test sets (20% of samples) and computing the mean of the AUC for the 5 permutations. The mean AUC for the complete model including the biomarker was compared with the mean AUC for the model without the biomarker as a covariate. Using the FFQ data for categorization of smoked meat intake, the same Welch 2-sample *t* test was performed. Similarly to 24HDR data, logistic regression models were built with biomarker intensities, personal data of participants, and habitual food intake as covariates. The same cross-validated ROC analysis was performed for the FFQ data. All statistical analyses were carried out using the R open-source statistical software, version 3.5.0 (R Foundation for Statistical Computing, Vienna, Austria).

## Results

### Metabolic profiles of in vitro meat digests

In vitro digests of 36 food products (12 fresh meat products, 21 processed meat products, and 3 tofu products; **Supplemental Table 3**) were analyzed by LC-MS and intensities of 4581 MS features were compared between different groups of meat products. A total of 2022 MS features showed significant

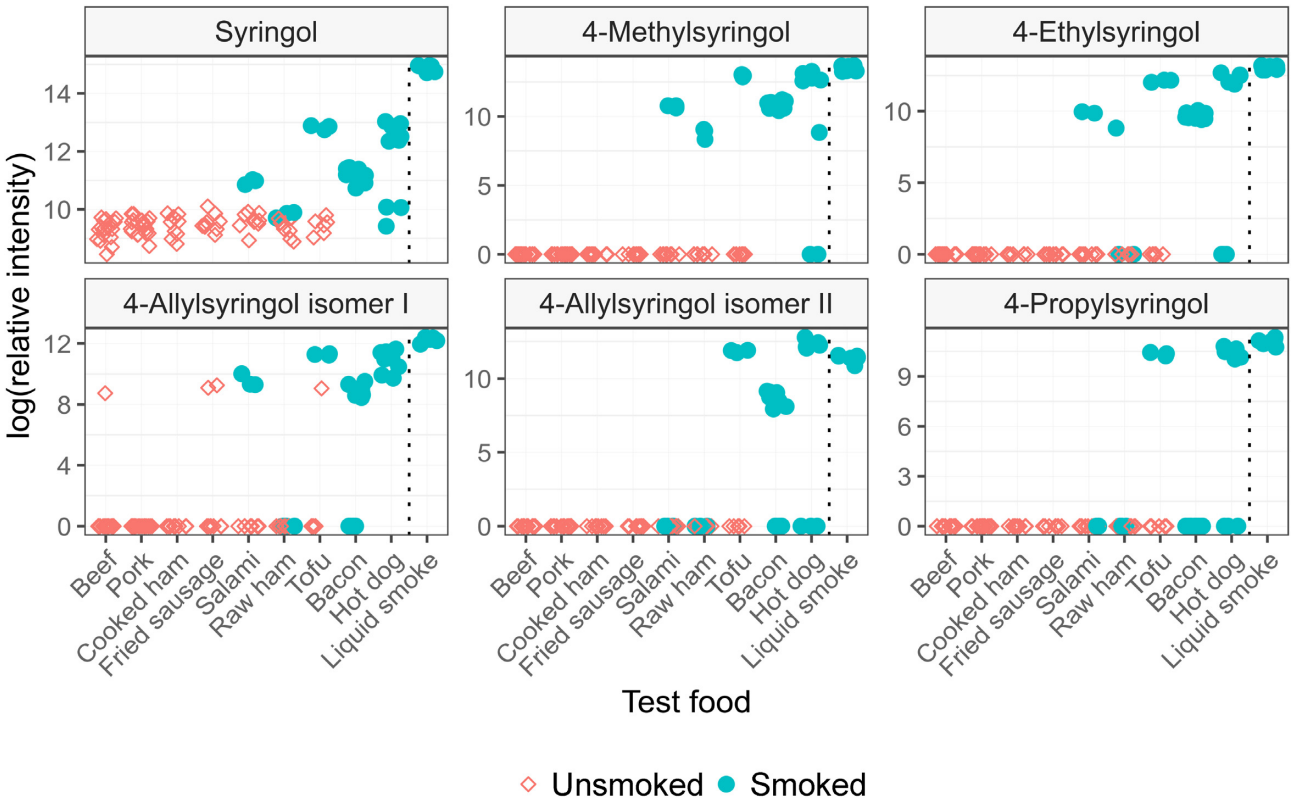


**FIGURE 1** Relative intensities of syringol and syringol derivatives in in vitro digests of 33 meat products and tofu.

differences in intensity between processed and nonprocessed meats. The features significantly elevated ≥2-fold in processed meat ($n$ = 178) formed several distinctive clusters when arranged by correlation of intensities (**Supplemental Figure 11**A). Compounds in 2 intercorrelated clusters highlighted in red in Supplemental Figure 11A showed a striking distribution in meat products. All were almost exclusively present in smoked processed meat (Supplemental Figure 11B). Some of these highly correlated compounds ($r$ = 0.77–0.99) could be identified as syringol (2,6-dimethoxyphenol) and several syringol derivatives including 4-methylsyringol, 4-ethylsyringol, 4-propylsyringol, and 4-allylsyringol (**Supplemental Table 4**). All these compounds also showed high intensities in a commercial extract of wood smoke analyzed for comparison (**Figure 1**). These compounds were further explored as possible biomarkers of smoked meat intake in a dietary intervention study and in a cross-sectional study.

### Biomarkers of smoked meat intake in a randomized crossover dietary intervention study

A randomized crossover dietary intervention study in which volunteers consumed 3 types of processed meat including 2 smoked meat products (hot dog and bacon) and 2 control foods was conducted to identify urinary and plasma biomarkers of smoked meat intake (Supplemental Figure 1).

The cumulative 12-h urine samples were analyzed by LC-MS, resulting in a data set of 12,624 MS features detected in ≥75% of samples in ≥1 of the intervention diet groups. To identify biomarkers of smoked meat intake, the cumulative 12-h urine samples collected after intake of the hot dog diet were compared with those collected after intake of pork. Three hundred and sixty-five MS features significantly differed in their intensities between groups, with 261 of those significantly elevated in hot dog diet samples compared with pork diet samples (**Supplemental Table 5**). Among the corresponding metabolites with the highest intensity, sulfate esters of syringol and syringol derivatives could be identified by comparison with in-house synthesized standards (**Figure 2**, **Supplemental Table 6**). None of these compounds were significantly elevated after bacon intake compared with pork intake after adjustment for multiple testing, although an increased trend was observed for syringol derivatives (Figure 2). Syringol metabolites were also elevated in fasting plasma samples taken 12–15 h after the last meal in subjects consuming smoked meats (**Supplemental Figure 12**).

In addition to the sulfated syringols, other urinary discriminants of smoked meats were annotated as the corresponding glucuronides (**Supplemental Table 7**). However, their intensities in urine were 1.3- to 14-fold lower than those of sulfated metabolites. Because of the higher intensities and unambiguous identification of sulfated metabolites, we focused on those as biomarkers of smoked meat intake in subsequent analyses.

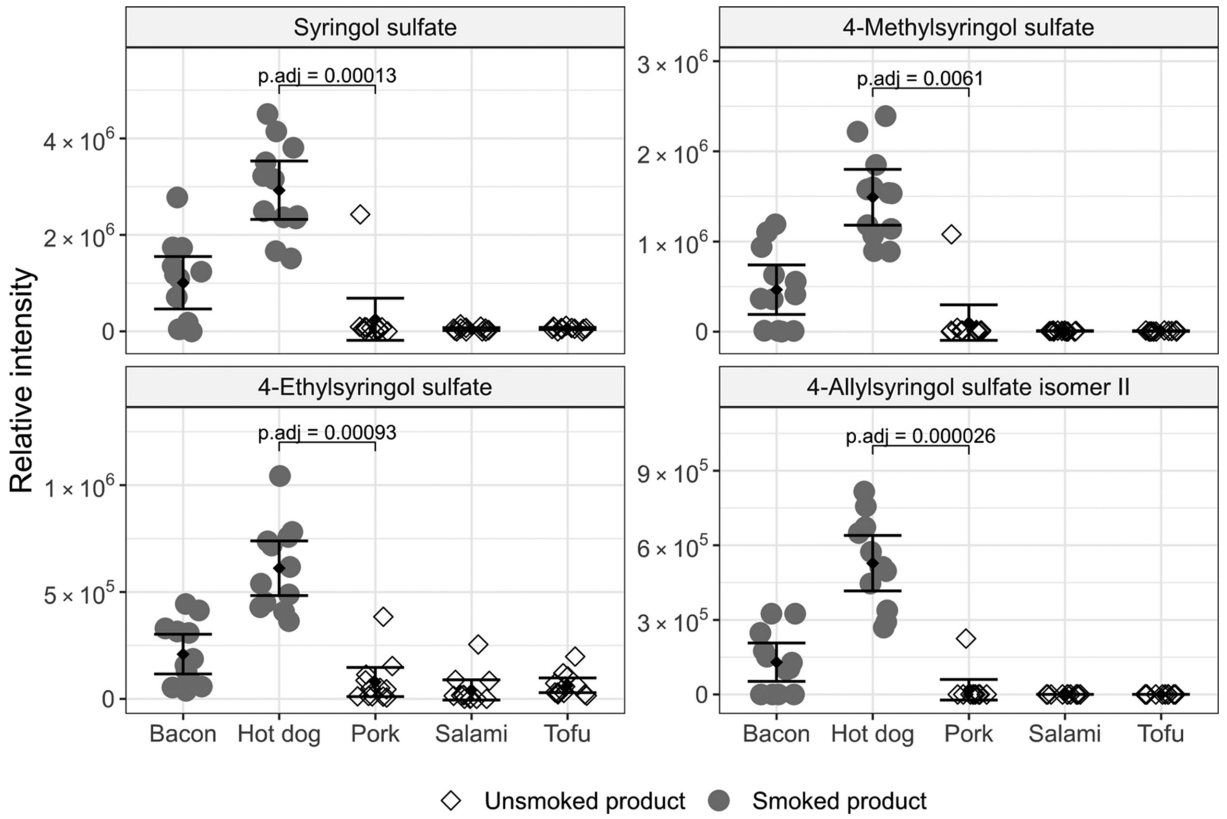


**FIGURE 2** Relative intensities of syringol sulfates in cumulative 12-h urine samples from the dietary intervention study. The figure shows relative intensity for every urine sample and the mean and 95% CI for each diet ($n = 12$ per diet). The adjusted $P$ value (p.adj.) for a paired Student's $t$ test comparing the relative intensities of urine samples after smoked meat intake (hot dog and bacon) and pork intake is indicated for each compound (correction for 12,624 tests using the Benjamini–Hochberg method with the false discovery rate set to 0.05). Differences between bacon and pork did not reach significance after correction for multiple testing. Unadjusted $P$ values were 0.093, 0.015, 0.055, and 0.031 for syringol sulfate, 4-methylsyringol sulfate, 4-ethylsyringol sulfate, and 4-allylsyringol sulfate, respectively.

A significant increase in the concentrations of syringol sulfates was also observed in spot urine samples collected 2 and 12 h after the first test meal consumed in the evening (**Supplemental Figure 13**). Mean relative intensities of most syringol sulfates decreased from 2 to 12 h after the meal with the exception of 4-allylsyringol sulfate, which showed a higher mean intensity at 12 h than at 2 h after the test meal.

### Biomarkers of smoked meat intake in the EPIC cross-sectional study

Biomarkers of smoked meat intake identified in the dietary intervention study were tested in 474 subjects from the EPIC cross-sectional study for whom urine samples and 24-HDR and dietary questionnaires were available. Large variations in the consumption of smoked meat were observed between countries, with the highest consumption found in Germany and lowest consumption in Greece and Italy (Table 1). Based on short-term intake data collected with 24HDRs, participants were categorized into nonconsumers of smoked meat, low-consumers (below the median intake of all consumers), and high-consumers (above the median intake of all consumers). The mean intensity of 4 syringol sulfate compounds in urine (**Figure 3**) was significantly higher in low-consumers than in nonconsumers and significantly higher in high-consumers than in low-consumers (Welch 2-sample $t$ test, $P < 0.05$).

To exclude the possibility of confounding by cigarette smoke, the same analysis was performed stratified by self-reported smoking status of the participants. The same patterns were observed with increases of urinary excretion of syringol sulfates according to smoked meat intake amounts for the different tobacco smoking status groups, as illustrated for 4-methyl syringol sulfate (**Supplemental Figure 14**). Possible confounding by other smoked foods was also examined. A significant increase in urinary excretion of 4-methylsyringol sulfate and 4-ethylsyringol sulfate was observed in recent consumers of smoked fish ($n = 15$) compared with volunteers that did not consume smoked foods as recorded in the 24HDR (**Supplemental Figure 15**).

To test for the influence of various potential confounders, a logistic regression model was built for every syringol sulfate compound that included a series of covariates related to study design (e.g., study center), participant characteristics (e.g., age, BMI, sex, smoking status), and habitual intake of different food groups as described in the FFQ (e.g., smoked fish, vegetables, fruits, fats) and as the outcome consumption or nonconsumption of smoked foods as reported in the 24HDR. For 4-methylsyringol sulfate, syringol sulfate, 4-ethylsyringol

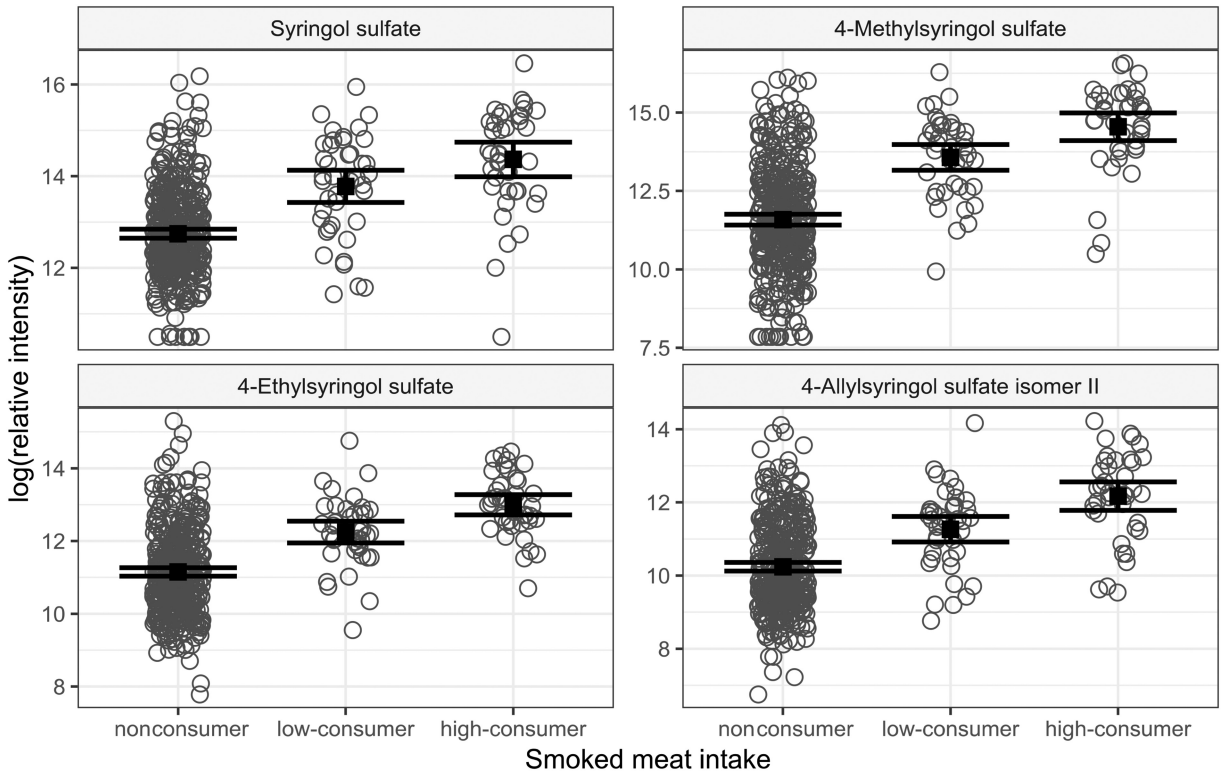


**FIGURE 3** Relative intensity of selected syringol sulfates in 24-h urine samples in 474 subjects from the European Prospective Investigation into Cancer and Nutrition cross-sectional study, stratified according to their short-term self-reported smoked meat intake (high-consumers, $n = 40$; low-consumers, $n = 40$; nonconsumers, $n = 394$). Means and 95% CIs are shown for each smoked meat consumer category. For all compounds, differences between the nonconsumer and low-consumer groups as well as between the low-consumer and high-consumer groups were statistically significant ($P < 0.05$) after correction for multiple testing using the Benjamini–Hochberg method with the false discovery rate set to 0.05.

sulfate, and 4-allylsyringol sulfate, the adjusted $P$ value of the biomarker intensity as a covariate was $3.19 \times 10^{-09}$, $6.86 \times 10^{-06}$, $1.71 \times 10^{-08}$, and $1.56 \times 10^{-05}$, respectively (**Supplemental Figure 16**). Adding the biomarker as a covariate in the model consistently increased the discriminatory power of the model as measured by an increased AUC, estimated through cross-validation. The ability of the biomarker alone to discriminate short-term smoked meat consumers from nonconsumers was further tested using ROC analyses. The AUCs ranged between 0.78 for 4-allylsyringol sulfate and 0.86 for 4-methylsyringol sulfate (**Figure 4**A).

A similar analysis was carried out on prediction of habitual intake of smoked meats using the FFQ data. High habitual consumers had significantly higher amounts than nonconsumers for all 4 syringol sulfates (**Supplemental Figure 17**). This was also the case when the data were stratified by cigarette smoking status (nonsmoker, former smoker, and current smoker). To test for other covariates that could predict consumption of smoked meat, logistic regression models were built that included other participant characteristics and habitual food intake. All 4 syringol compound intensities in urine were significant covariates in models predicting habitual intake of smoked meats. ROC curves for the prediction of habitual intake of smoked meats displayed AUCs of up to 0.79 for 4-methylsyringol sulfate (Figure 4B).

## Discussion

In this work, a fully agnostic multi-tiered metabolomics approach was used to identify novel biomarkers for smoked meat intake. Four syringol compounds were found in meat digests to be strong discriminants of smoked meat products when compared with nonsmoked meat products. In the dietary intervention study, concentrations of sulfate metabolites of the same compounds were also increased in urine and plasma of consumers of smoked meat when compared with nonsmoked meat products. Finally, the same compounds showed a significant increase in urinary excretion in free-living subjects from the EPIC cross-sectional study reporting recent and habitual consumption of smoked meat. The syringol sulfate compounds were found to be good predictors of smoked meat intake in the population tested.

Syringol and its related compounds are combustion products of lignins, phenolic polymers found primarily in hardwood. They are major constituents of wood smoke condensate and known constituents of smoked meats and other smoked products (18–21). In humans, syringol, once absorbed, is rapidly conjugated and excreted as glucuronide or sulfate conjugates (22). Several methoxy phenols (mainly guaiacols and syringols measured in urine after enzymatic deconjugation) have been proposed as biomarkers of exposure to inhaled wood smoke and were also found to be elevated in the urine of 3 volunteers after ingestion of

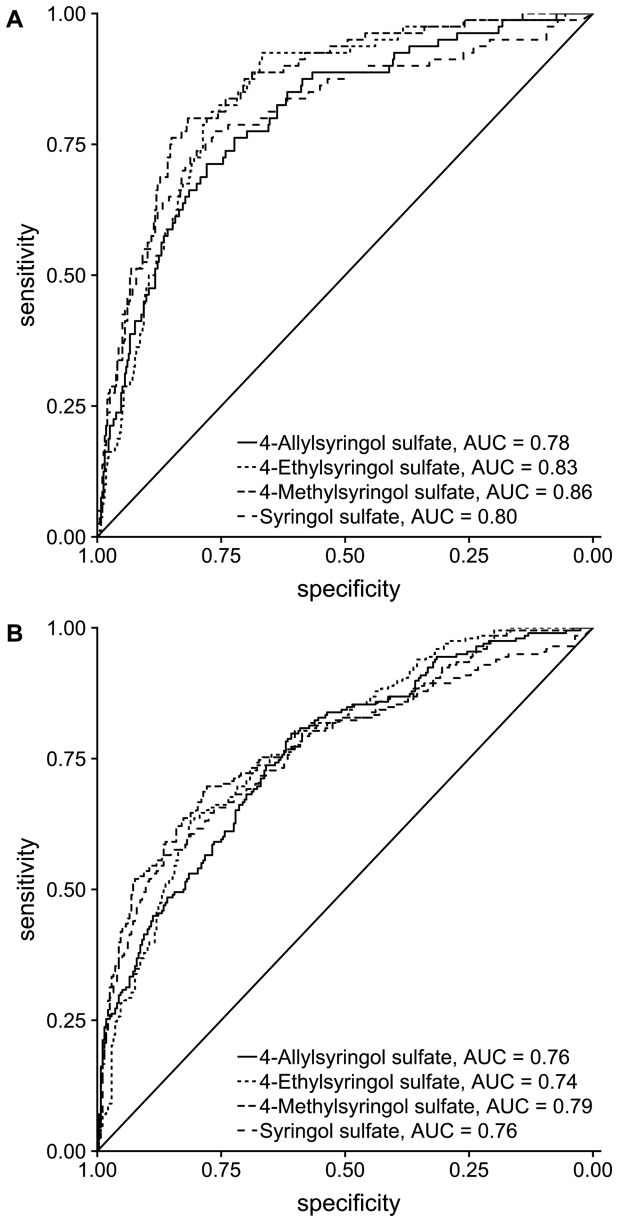


**FIGURE 4** Receiver operating characteristic analysis in 474 subjects from the European Prospective Investigation into Cancer and Nutrition cross-sectional study for concentrations of urinary sulfated syringol and syringol derivatives as predictors of smoked meat consumption. (A) Prediction of short-term consumers based on 24-h dietary recall data. (B) Prediction of habitual consumers based on FFQ data. AUCs are shown for all 4 metabolites. Prediction was done using a logistic regression model with biomarker concentrations as covariates. The AUC for prediction of short-term smoked meat intake was significantly higher for 4-methylsyringol sulfate than for syringol sulfate and 4-allylsyringol sulfate. No other differences in AUC were found to be statistically significant.

a dose of liquid smoke (23). To our knowledge, our study is the first to identify specific biomarkers of smoked meat consumption in urine and blood.

The half-life of syringol compounds in humans tends to be short (22), but all compounds tested were still detected in blood and urine 12 h after consumption of smoked foods. Their urinary excretion was not only correlated with short-term intake of smoked meat, but also with their habitual intake. This may be explained by a sufficient retention of syringol compounds in the body. All compounds still showed an elevated concentration in spot urine samples collected 12 h after consumption of smoked meat. Associations of syringol compounds in urine with habitual smoked meat intake in EPIC could also be explained by the large heterogeneity in smoked meat consumption throughout the different EPIC countries involved and a relatively frequent consumption of smoked meat in Germany, from where most of the smoked meat consumers included in the EPIC-part of the present study originate and where 94% of participants consumed smoked meat habitually (Table 1).

In our analysis, the role of possible confounders was also tested. Cigarette smoking had no effect on the associations of syringol compounds with smoked meat intake in urine. Syringol compounds were found in smoked soy products (Figure 1) and consumption of smoked fish increased concentrations of the syringol compounds in the EPIC cross-sectional study (Supplemental Figure 7). The number of consumers of these particular smoked foods was low among the 474 subjects in the cross-sectional study (Table 1) and thus confounding by these foods is unlikely or limited in our study population (Supplemental Figure 7). Nevertheless, the biomarkers' use in other populations will require examination of potential confounding by other smoked food products when investigating associations with smoked meat intake.

Smoked meat biomarkers may help in exploring the role of smoked meat products in cancer etiology, and more particularly their contribution to the risk of colorectal cancer. Indeed, estimation of intake of different processed meat products is often difficult owing to the lack of sufficient details on these foods in many dietary questionnaires. Biomarkers of smoked meat intake could be used as a valuable complementary tool to quantify the consumption of this food group in epidemiological studies on cancer risk. A high consumption of smoked meats in Germany and Northern Europe of $\leq$30 g/d in women and $\leq$50 g/d in men that was reported by Linseisen et al. (24) shows that this food group should be more thoroughly assessed when searching for associations of intake of different meat products and cancer risk.

Syringol sulfates have been found to be nongenotoxic after nitrosation (25). However, smoked meat products also contain known carcinogenic compounds such as PAHs, NOCs, and heterocyclic amines (26). Some of these compounds are also present in other processed meat products and a better estimate of the intake of different processed meat products should help to disentangle their respective contributions to cancer risk in prospective observational studies.

Our study has several strengths. First, the multi-tiered metabolomics approach allows the identification of the most discriminant factors in a fully agnostic way. It combines analysis of liquid smoked extracts, an in vitro digestion of processed meat, a dietary intervention study, and the validation of biomarkers in a cross-sectional study and shows great consistency between all results. The EPIC cross-sectional study was found to be ideal for validation purposes because of the large heterogeneity of dietary habits in the different European countries. Both dietary recall and FFQ data were also available, allowing the evaluation of associations of biomarkers with both short-term and habitual food intake, showing that in this particular population, syringol compounds were able to discriminate habitual consumers from nonconsumers. Lastly, a series of potential confounders were

tested, showing the absence of major confounders in the population studied, but also emphasizing possible confounding by other smoked food products such as smoked fish.

This study also has a number of limitations. Data on smoked meat intake in the cross-sectional study are incomplete. For some meat products, data on food processing such as smoking were lacking and smoked and nonsmoked products were grouped together. Some consumers might have thus been misclassified as nonconsumers and this may explain elevated biomarker concentrations in some nonconsumers (Figure 3). Another limitation is that we only replicated the findings in the cross-sectional study with 24-h urine samples, and not with spot urine samples which were not available, or with plasma samples because of insufficient sensitivity of the broad-scan MS method used. We also cannot exclude some possible degradation of sulfate esters of syringol and syringol derivatives during storage of urine samples at −20°C. However, we were able to find sulfate esters of diverse polyphenols at high concentrations in the same urine samples after such long storage at −20°C (27). The fact that we could measure syringol sulfates and that they successfully discriminated consumers from nonconsumers of various foods made us confident that they could be used as markers even after long-term storage of urine samples. A last limitation is that findings of this study would need to be replicated in other populations which may also consume types of smoked foods other than meat such as smoked fish or cheese (28).

In conclusion, several new biomarkers of smoked meat intake were identified in plasma and urine and replicated in urine samples of a population-based study. The results suggest that the identified biomarkers of smoked meat intake could be used in epidemiological studies to improve classification of participants according to their consumption of smoked meat products and to study the role of smoked meat products as risk factors for diseases such as cancer.

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## References

1. Bouvard V, Loomis D, Guyton KZ, Grosse Y, Ghissassi FE, Benbrahim-Tallaa L, Guha N, Mattock H, Straif K. Carcinogenicity of consumption of red and processed meat. Lancet Oncol 2015;16:1599–600.
2. Rohrmann S, Overvad K, Bueno-de-Mesquita HB, Jakobsen MU, Egeberg R, Tjønneland A, Nailler L, Boutron-Ruault M-C, Clavel-Chapelon F, Krogh V, et al. Meat consumption and mortality – results from the European Prospective Investigation into Cancer and Nutrition. BMC Med 2013;11:63.
3. Micha R, Michas G, Mozaffarian D. Unprocessed red and processed meats and risk of coronary artery disease and type 2 diabetes – an updated review of the evidence. Curr Atheroscler Rep 2012;14:515–24.
4. Heinz G, Hautzinger P. Meat processing technology for small- to medium-scale producers [Internet]. Bangkok: Food and Agriculture Organization of the United Nations Regional Office for Asia and the Pacific; 2007 [cited 3 November, 2016]. Available from: http://www.fao.org/3/a-ai407e.pdf.
5. Micha R, Khatibzadeh S, Shi P, Andrews KG, Engell RE, Mozaffarian D; Global Burden of Diseases Nutrition and Chronic Diseases Expert Group (NutriCoDE). Global, regional and national consumption of major food groups in 1990 and 2010: a systematic analysis including 266 country-specific nutrition surveys worldwide. BMJ Open 2015;5:e008705.
6. Linseisen J, Kesse E, Slimani N, Bueno-de-Mesquita HB, Ocké MC, Skeie G, Kumle M, Iraeta MD, Gómez PM, Janzon L, et al. Meat consumption in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohorts: results from 24-hour dietary recalls. Public Health Nutr 2002;5:1243–58.
7. Cross AJ, Sinha R. Meat-related mutagens/carcinogens in the etiology of colorectal cancer. Environ Mol Mutagen 2004;44:44–55.
8. Jenab M, Slimani N, Bictash M, Ferrari P, Bingham SA. Biomarkers in nutritional epidemiology: applications, needs and new horizons. Hum Genet 2009;125:507–25.
9. Almeida AF, Santos CN, Ventura MR. Synthesis of new sulfated and glucuronated metabolites of dietary phenolic compounds identified in human biological samples. J Agric Food Chem 2017;65:6460–6.
10. Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, Carrière F, Boutrou R, Corredig M, Dupont D, et al. A standardised static *in vitro* digestion method suitable for food – an international consensus. Food Funct 2014;5:1113–24.
11. Tollosa DN, Van Camp J, Huybrechts I, Huybregts L, Van Loco J, De Smet S, Sterck E, Rabâi C, Van Hecke T, Vanhaecke L, et al. Validity and reproducibility of a food frequency questionnaire for dietary factors related to colorectal cancer. Nutrients 2017;9:1257.
12. Riboli E, Hunt KJ, Slimani N, Ferrari P, Norat T, Fahey M, Charrondière UR, Hémon B, Casagrande C, Vignat J, et al. European Prospective Investigation into Cancer and Nutrition (EPIC): study populations and data collection. Public Health Nutr 2002;5:1113–24.
13. Edmands WMB, Ferrari P, Scalbert A. Normalization to specific gravity prior to analysis improves information recovery from high resolution mass spectrometry metabolomic profiles of human urine. Anal Chem 2014;86:10925–31.
14. Robinson O, Keski-Rahkonen P, Chatzi L, Kogevinas M, Nawrot T, Pizzi C, Plusquin M, Richiardi L, Robinot N, Sunyer J, et al. Cord blood metabolic signatures of birth weight: a population-based study. J Proteome Res 2018;17:1235–47.
15. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, Brandon TR, Custodio DE, Abagyan R, Siuzdak G. METLIN: a metabolite mass spectral database. Ther Drug Monit 2005;27:747–51.
16. Wishart DS, Feunang YD, Marcu A, Guo AC, Liang K, Vázquez-Fresno R, Sajed T, Johnson D, Li C, Karu N, et al. HMDB 4.0: the human metabolome database for 2018. Nucleic Acids Res 2018;46:D608–17.
17. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, Fan TW-M, Fiehn O, Goodacre R, Griffin JL, et al. Proposed minimum reporting standards for chemical analysis: Chemical Analysis Working Group (CAWG) Metabolomics Standards Initiative (MSI). Metabolomics 2007;3:211–21.
18. Knowles ME, Gilbert J, McWeeny DJ. Phenols in smoked cured meats. Phenolic composition of commercial liquid smoke preparations and derived bacon. J Sci Food Agric 1975;26:189–96.
19. Guillen MD, Manzanos MJ, Zabala L. Study of a commercial liquid smoke flavoring by means of gas chromatography/mass spectrometry and Fourier transform infrared spectroscopy. J Agric Food Chem 1995;43:463–8.
20. Montazeri N, Oliveira ACM, Himelbloom BH, Leigh MB, Crapo CA. Chemical characterization of commercial liquid smoke products. Food Sci Nutr 2013;1:102–15.
21. Varlet V, Knockaert C, Prost C, Serot T. Comparison of odor-active volatile compounds of fresh and smoked salmon. J Agric Food Chem 2006;54:3391–401.
22. Ogata N, Matsushima N, Shibata T. Pharmacokinetics of wood creosote: glucuronic acid and sulfate conjugation of phenolic compounds. Pharmacology 1995;51:195–204.
23. Dills RL, Zhu X, Kalman DA. Measurement of urinary methoxyphenols and their use for biological monitoring of wood smoke exposure. Environ Res 2001;85:145–58.

24. Linseisen J, Rohrmann S, Norat T, Gonzalez CA, Iraeta MD, Gómez PM, Chirlaque M-D, Pozo BG, Ardanaz E, Mattisson I, et al. Dietary intake of different types and characteristics of processed meat which might be associated with cancer risk – results from the 24-hour diet recalls in the European Prospective Investigation into Cancer and Nutrition (EPIC). Public Health Nutr 2006;9: 449–64.
25. Ohshima H, Friesen M, Malaveille C, Brouet I, Hautefeuille A, Bartsch H. Formation of direct-acting genotoxic substances in nitrosated smoked fish and meat products: identification of simple phenolic precursors and phenyldiazonium ions as reactive products. Food Chem Toxicol 1989;27:193–203.
26. Kazerouni N, Sinha R, Hsu CH, Greenberg A, Rothman N. Analysis of 200 food items for benzo[a]pyrene and estimation of its intake in an epidemiologic study. Food Chem Toxicol 2001;39:423–36.
27. Edmands WM, Ferrari P, Rothwell JA, Rinaldi S, Slimani N, Barupal DK, Biessy C, Jenab M, Clavel-Chapelon F, Fagherazzi G, et al. Polyphenol metabolome in human urine and its association with intake of polyphenol-rich foods across European countries. Am J Clin Nutr 2015;102:905–13.
28. Clark M, Paulsen M, Smith KR, Canuz E, Simpson CD. Urinary methoxyphenol biomarkers and woodsmoke exposure: comparisons in rural Guatemala with personal CO and kitchen CO, levoglucosan, and $PM_{2.5}$. Environ Sci Technol 2007;41:3481–7.