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# Linalool, 1,8-Cineole, and Eugenol Transfer from a Curry Dish into Human Urine

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**Scope: For most substances, there are several routes of excretion from the human body. This study focuses on urinary excretion of dietary odorants and compares the results with previously obtained results on excretion into milk.**
**Methods and results: Lactating mothers ($n = 18$) are given a standardized curry dish and donate urine samples before and after the intervention. The odorants 1,8-cineole, linalool, cuminaldehyde, cinnamaldehyde, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, sotolone, eugenol, vanillin, and $\gamma$-nonalactone are quantitatively analyzed. A significant transition of up to 6 µg $g^{-1}$ creatinine into urine is observed for linalool, 1,8-cineole, and eugenol. Maximum concentrations are reached 1.5 h after the intervention for 1,8-cineole and eugenol as well as 2.5 h after the intervention for linalool. Comparison with previous results reveals that the excretion pattern of odorants into urine is divergent from the one into milk. In a second intervention study ($n = 6$), excretion of phase II metabolites into urine is studied using $\beta$-glucuronidase treatment. Linalool and eugenol concentrations are 23 and 77 times higher after treatment than before treatment with $\beta$-glucuronidase, respectively.**
**Conclusion: The study demonstrates transition of linalool, 1,8-cineole, and eugenol from the diet into urine and excretion of glucuronides in the case of linalool, eugenol, and vanillin.**

## 1. Introduction

The analysis of bodily fluids such as blood or urine has long been a standard procedure in medical diagnostics for the detection of diseases or for tracking the intake of restricted substances.[1] Likewise, it is a valuable tool for researchers to gain insights into the uptake, biotransformation, and excretion of various potentially bioactive substances ingested with our daily food intake.[2] A particularly precious body fluid in this context is breast milk, as it is only available to a very limited extent in terms of time and quantity. Breast milk is also of particular interest because it is the newborn's first and often only food for several months. Accordingly, chemosensory impressions conveyed by breast milk have been proposed to be formative for the infant.[3] We recently demonstrated that flavor substances from a curry dish, notably linalool, can be transferred into the mother's milk in relevant amounts.[4] Within this study it was shown, that linalool transferred into milk already within 1 h and that this transition can be perceived by the human nose. For the other investigated aroma compounds (1,8-cineole, cuminaldehyde, cinnamaldehyde, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDF), sotolone, eugenol, vanillin, and $\gamma$-nonalactone), no significant transfer into milk became evident. The excretion of aroma compounds via breast milk is only one possible excretion pathway next to others, such as via urine, breath, or feces. In general, dietary odorant uptake occurs via mucous membranes within the digestive tract and lung. The

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odorants can then be transported via the blood stream to the alveoli of the mammary glands, enabling potential excretion via milk,[5] to the kidney, enabling potential excretion via urine,[6] or to the lung, enabling potential excretion via breath. Moreover, irrespective of the medium of excretion, it is well known that a substantial amount of the ingested aroma compounds can undergo biotransformation in the human body and be excreted in the form of its derivatives.[7] Especially for the excretion into urine, conjugation with glucuronic acid, amongst other phase II conjugates like sulfates or mercapturic acids, plays an important role in the metabolism of xenobiotics.[8]

With the present experiments, the aim was to obtain a deeper understanding of the metabolic fate and excretion of aroma compounds ingested with a standardized curry dish. Therefore, the concentrations of nine target compounds in the urine of the mothers who participated in the intervention study on flavor transfer into milk were monitored.[4] The results are reported here and compared with the results obtained for milk.[4] Moreover, a second intervention study with non-lactating women was conducted to obtain further insights into the extent of glucuronidation of the same target compounds and the excretion of such glucuronides into urine.

## 2. Experimental Section

### 2.1. Chemicals

Linalool, 1,8-cineole, eugenol, cinnamaldehyde, vanillin, cuminaldehyde, HDF, sotolone, and $\gamma$-nonalactone were purchased from Sigma Aldrich (Steinheim, Germany). The corresponding isotopically labeled standards $^{2}H_{4-5}$-linalool, $^{2}H_{3}$-1,8-cineole, $^{2}H_{3}$-eugenol, $^{2}H_{6}$-cinnamaldehyde, $^{2}H_{8}$-cuminaldehyde, $^{13}C_{2}$-HDF, $^{13}C_{2}$-sotolone, and $^{2}H_{4}$-$\gamma$-nonalactone were purchased from aromaLAB GmbH (Martinsried, Germany) while $^{13}C_{6}$-vanillin was bought from Sigma Aldrich. Sodium sulfate and dichloromethane HiPerSolv (DCM) were from VWR International GmbH (Ismaning, Germany). The DCM was freshly distilled prior to usage. Sigma Aldrich provided $\beta$-glucuronidase helix pomatia, type HP-2 with an activity of $\geq$100 000 units mL$^{-1}$, and sodium azide, Th. Geyer GmbH & Co. KG (Renningen, Germany) provided acetic acid $\geq$99.5%, and Merck KGaA (Darmstadt, Germany) sodium acetate. A sodium hydroxide solution, a picrinic acid solution as well as a creatinine solution were from Labor + Technik Eberhard Lehmann GmbH (Berlin, Germany) as part of the kit LT-SYS creatinine (y).

### 2.2. Participants

Two experiments were conducted. In the first experiment, during which milk samples were collected as well,[4] the urine samples were provided by 18 lactating mothers (mean age: 32 $\pm$ 2 years). The urine samples of three of these mothers were used for an initial screening using gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS) while the samples of another two mothers were used for adjusting the optimum quantity of isotopically labeled standards. The samples of the remaining 13 mothers (labeled from A to M) were used for quantification. In the second experiment, which was conducted to study the excretion of phase II conjugates via urine, six non-lactating women participated (mean age: 26 $\pm$ 3 years). Only healthy (neither chronic nor acute diseases), and non-smoking persons with no allergies to the ingredients of the curry dish were included in the study. This was ascertained in advance of the study by means of questionnaires. All participants gave their informed written consent prior to participating in the study. Withdrawal from the study was possible at any time without negative consequences. The study was designed in accordance with the Declaration of Helsinki, while the ethical committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg approved the study protocol (registration number 24_16 B).

### 2.3. General Description of the Intervention Studies and Analytical Methods

A curry dish was prepared and used for the intervention studies as described in detail in Debong et al.[4] and N'Diaye et al. (2021). Briefly, the participants underwent a 2-day wash-out phase and ate a standardized breakfast (porridge or white bread with butter/margarine and non-flavored strawberry jam) on the sampling day. They donated one sample before and three samples after consumption of the curry dish. The curry dish weighed on average 246 g and consisted of rice with a sauce made of coconut milk, water, sunflower oil, fresh ginger, salt, and curry powder. The ingredients of the curry powder were coriander seeds, fenugreek, cumin seeds, peppercorns, dried red chilies, cinnamon sticks, cardamom, cloves, and curry leaves. The average amounts of the target compounds per dish were: linalool (35 mg), 1,8-cineole (0.4 mg), eugenol (1.1 mg), cinnamaldehyde (3.6 mg), vanillin (0.5 mg), cuminaldehyde (22 mg), sotolone (3 µg), HDF (<34.36 ng [limit of detection, LOD]), and $\gamma$-nonalactone (8 µg), see Debong et al. (2021) for details. Aroma extracts of the donated urine samples were obtained using solvent extraction followed by solvent assisted flavor evaporation (SAFE)[9] and analyzed by GC-O and GC-MS. For quantification, a stable isotope dilution assay (SIDA approach)[10] was used. For more details on LOD, limits of quantification (LOQ) determination, and calibration curves see Debong et al. (2021) as well as Table S1, Supporting Information. Procedures that were specific to the here presented experiments were detailed in the following.

#### *2.3.1. Sampling of Urine*

Urine samples were collected 1 h before (U1) and 0.5, 1.5, and 2.5 h after (U2-4) consumption of the curry dish, which took place at 12 o'clock. The amount of urine donated per sample was 50 $\pm$ 16 mL in Experiment 1, and 64 $\pm$ 6 mL in Experiment 2. The participants were asked to donate midstream urine (spot urine sampling), and only used the lavatories for the sampling. Therefore, the urine excretion overall was higher than what was donated and samples were normalized by their creatinine content (see Section 2.8). Aliquots (2 mL) of each urine sample were taken for the investigation of tastants and non-volatiles within a partner project (N'Diaye et al., in preparation) as well as for the determination of the creatinine content of the samples. The sam-

ples were stored in brown glass bottles at −80 °C for up to 30 days prior to analysis.

#### 2.3.2. Isolation of Volatiles from the Urine Samples

First, DCM was added to the urine samples in a ratio of 1:1 (DCM:urine, v/v), followed by the addition of 100 µL of a mixture of the respective isotopically labeled standards (see Table S1, Supporting Information) and a stirring time of 30 min at room temperature and 300 rpm. Afterwards, the organic phase was separated from the aqueous phase and subjected to SAFE at 50 °C and a pressure of $10^{-4}$ mbar. Within the SAFE procedure, the volatile fraction of the sample was cryotrapped by liquid nitrogen. The distilled samples were thawed, dried over anhydrous sodium sulfate, and concentrated. The concentration steps were performed by Vigreux and micro distillation to a final volume of 100 µL.

### 2.4. Glucuronidase-Assay (Experiment 2)

Each urine sample obtained from Experiment 2 was divided into two parts. One part was treated with $\beta$-glucuronidase and the other one was spiked with the respective amount of distilled water as a control. Specifically, aliquots of 10 mL of the urine samples were added to 10 mL of an acetic acid/sodium acetate buffer with a pH of 5.0. Subsequently, 0.2 mL of $\beta$-glucuronidase solution (100 000 u $mL^{-1}$) in the case of the treated aliquots, or 0.2 mL of distilled water in the case of the control aliquots, followed by 0.1 mL of a sodium azide solution (200 µg $mL^{-1}$) and isotopically-labeled standards were added. The mixture was then stirred in a water bath at 37 °C for 15 h. After stirring, the samples were prepared for analysis as described in Section 2.3.

### 2.5. Untargeted Screening for Odorant Transition and Selection of Target Compounds

The distillates of three urine sets obtained from Experiment 1 were initially screened by GC-O and GC-MS to evaluate the transfer of odorants into urine through perceivable or obvious quantitative differences in the samples before (U1) and after (U2-4) consumption of the curry dish. The retention indices on two columns, the perceived odor quality, and the mass spectrum were compared to those of analytical standards for identification. The screening comprised an aroma extract dilution assay (AEDA), which was performed by two panelists with dilution steps of 1:3 v/v. The main odorants were determined as those perceivable at the GC-O at the highest factors of dilution (flavor dilution factor, FD factor). All distillates and their dilutions were analyzed on two different capillary columns (DB-5 and DB-FFAP). A homologous series of n-alkanes from C5 to C34 was injected to determine retention indices according to Kovats.[11] These GC-O measurements revealed a higher FD factor for eugenol after the ingestion of the dish (not perceivable at FD 1 for U1 and perceivable up to FD 27 for U3). No further differences became apparent. Additionally, the aroma extracts were analyzed by GC-MS. The data obtained were evaluated using a manual comparison of all chromatogram peaks with a signal-to-noise ratio ≥3 to discover potential target substances. For the substances linalool, 1,8-cineole, and eugenol, visible differences between the samples U1 and U2-4 were discovered. No further differences were apparent. Based on this screening and the initial screening of the milk samples and characterization of the curry dish,[4a] the following target compounds were chosen for quantification: linalool, 1,8-cineole, eugenol, cinnamaldehyde, vanillin, cuminaldehyde, HDF, sotolone, and $\gamma$-nonalactone.

The same manual procedure was carried out to check for release of odorants or their phase-I metabolites in glucuronidase-treated extracts of all samples of the first two participants. No obvious further transitions of odorants or their metabolites emerged. Therefore, no additional compounds were added to the target compounds.

### 2.6. Determination of Odor Activity Values

In order to provide an estimate of the extent to which food intake affects body fluids, in this case urine, odor activity values (OAV)[12] were calculated. The odor thresholds of the target substances had been determined previously (Debong et al., 2021). The OAV was calculated by dividing the determined odorant concentration in the urine sample (in µg $L^{-1}$) by the respective odor threshold in distilled water. An odorant with an OAV > 1 was assumed to be perceivable.

### 2.7. Determination of the Creatinine Content of Urine Samples

The creatinine content of the urine samples was determined by the Jaffé reaction, a stochastic color reaction of creatinine with picric acid, measured by a UV–vis photometer (Jasco V630, Jasco Deutschland GmbH, Pfungstadt, Germany). Following the procedure of LT-SYS creatinine (y), the urine samples were diluted 1:20 with distilled water prior to analysis. First, 100 µL of a standard solution containing 2 mg $L^{-1}$ creatinine was mixed with 500 µL of a 400 mM sodium hydroxide solution and 500 µL of a 55 mM picric acid solution. This mixture was measured at 20 °C and 492 nm in a square tube with a 1 cm side length at 20 s (E1) and 80 s (E2) after the mixing. Second, 100 µL of the diluted urine samples were measured analogously. The creatinine content was then calculated by the following formula as given in the kit's instructions:

$$C\ (\text{sample}) = 2\,\text{mg}\,\text{L}^{-1}\,C\,(\text{standard}) \times \frac{\Delta E\,(\text{sample})}{\Delta E\,(\text{standard})} \times 20 \quad (1)$$

$$C = \text{creatinine content} \quad (2)$$

$$\Delta E = \text{absorption difference} \quad (3)$$

This procedure was conducted in duplicate, setting the arithmetic mean as the final creatinine content of the urine sample.

### 2.8. Statistics

The effect of consumption of a standardized curry dish on the concentrations of the target compounds in urine was evaluated

for each compound by a univariate analysis of variance (ANOVA), followed by Dunnett's post hoc test. The data set of the samples obtained before consumption of the curry dish was used as a control set to check for significant differences against the three intervention sets after the curry dish consumption. Values <LOQ were factored in as their calculated value, and values <LOD were factored in as 0. The effect of the treatment of the samples with $\beta$-glucuronidase was evaluated for each odor compound by a Wilcoxon test. The data set of the untreated samples was tested against the data set of the treated samples to test for significant differences between the concentrations of these two sets. The data obtained from Experiment 1 and Experiment 2 were analyzed separately.

### 2.9. Determination of Transition Rates

To estimate the absolute amounts excreted into urine and to compare these to those of milk, transition rates were calculated by adding up the absolute amounts of a substance in the samples donated after the intervention (samples U2, U3, U4 in the case of urine and MM2, MM3, MM4 in the case of milk) and subsequent division by the respective dosage amount in the curry dish (See Section 2.3). Thereby, the concentration values of all participants were taken into account with concentrations >LOD being calculated in unchanged and those <LOD as 0. Given the fact that i) for neither milk nor for urine the total volume was sampled during the study, ii) mothers were allowed to breastfeed their infants during the study, this calculation serves as a rough estimation.

## 3. Results

### 3.1. Experiment 1: Excretion of the Target Compounds via Urine in Lactating Mothers

#### *3.1.1. Quantification of the Target Compounds in the Urine Samples*

The concentrations of the target substances in every sample are displayed in Table S2, Supporting Information as μg $g^{-1}$ creatinine and in Table S3, Supporting Information in μg $L^{-1}$ (for the amounts in the curry dish, see Section 2.3). Linalool and 1,8-cineole were detected in almost all control samples donated before consumption of the curry dish. The linalool concentration in these samples (U1) ranged from below the LOD ($n = 3$; participants D, F, M) to 0.69 μg $g^{-1}$ creatinine (average: 0.21 μg $g^{-1}$ creatinine, including values <LOD and <LOQ). The concentration of 1,8-cineole ranged from below the LOD ($n = 1$; H) to 0.79 μg $g^{-1}$ creatinine (average: 0.28 μg $g^{-1}$ creatinine). Vanillin was detected in the control samples of three mothers (A, F, M), with concentrations of up to 0.31 μg $g^{-1}$ creatinine (average: 0.12 μg $g^{-1}$ creatinine). Cuminaldehyde and eugenol were detected in one control sample each, with a concentration of 1.13 μg $g^{-1}$ creatinine (D) and 0.10 μg $g^{-1}$ creatinine (M), respectively. The concentrations of cinnamaldehyde, sotolone, HDF, and $\gamma$-nonalactone were below the LOD in all control samples and also in the other samples (U2, U3, U4).

After consumption of the curry dish, the concentrations of linalool rose to 3.89 μg $g^{-1}$ creatinine (ranges: <LOD–1.99 μg $g^{-1}$ creatinine for U2, 0.07–3.21 μg $g^{-1}$ creatinine for U3, and <LOD–3.89 μg $g^{-1}$ creatinine for U4). The concentrations of linalool were above the LOQ in all samples after intervention, except for samples U2 and U4 of participant M and sample U2 of participant F. The concentrations of 1,8-cineole increased up to 3.89 μg $g^{-1}$ creatinine (ranges: <LOD–1.24 μg $g^{-1}$ creatinine for U2, <LOD–2.57 μg $g^{-1}$ creatinine for U3, <LOD–3.89 μg $g^{-1}$ creatinine for U4). They were above the LOQ in all samples after intervention, except for samples U2–U4 of participants H and M and sample U2 of participant A. For eugenol, concentrations of up to 6.44 μg $g^{-1}$ creatinine were determined in the samples obtained after consumption of the curry dish (ranges: <LOD–0.65 μg $g^{-1}$ creatinine for U2, <LOQ–6.44 μg $g^{-1}$ creatinine for U3, <LOD – 5.47 μg $g^{-1}$ creatinine for U4). The concentrations of eugenol were above the LOD only in part of the samples U2 (B, F, K, L, and M), and above the LOQ in almost all samples U3 and U4, except for the samples U3 from participants C, G, and E and the samples U4 from participants B, C, G, H, and J. The concentrations of vanillin reached a maximum of 2.86 μg $g^{-1}$ creatinine (ranges: <LOD–1.78 μg $g^{-1}$ creatinine for U2, <LOD–2.86 μg $g^{-1}$ creatinine for U3, <LOD–1.57 μg $g^{-1}$ creatinine for U4). The concentrations of vanillin were above the LOQ for U2, U3, and U4 of participants A and M and above the LOD in samples U2 and U3 of participant F. The control samples of these three mothers also contained vanillin. The concentrations of cuminaldehyde rose to 7.38 μg $g^{-1}$ creatinine (ranges: <LOD–7.38 μg $g^{-1}$ creatinine for U2, <LOD–3.60 μg $g^{-1}$ creatinine for U3, <LOD–5.13 μg $g^{-1}$ creatinine for U4). Cuminaldehyde was detected above the LOD in one of the samples U2 (D), as well as above the LOQ in the samples U3 and U4 of seven participants (B, C, D, F, I, K, L). It was also detected in the control sample of participant D. The concentrations of the nine target substances in the urine samples, before and after the intervention, are presented in **Figure 1**.

The distributions of the concentrations of linalool, 1,8-cineole, eugenol, and cuminaldehyde across participants are presented in **Figure 2**. The concentration levels of the other target substances were either not or only for three participants above the LOQ. Therefore, no boxplot graphics are shown for these compounds.

For linalool, 1,8-cineole, and eugenol, the ANOVA demonstrated a significant impact of sampling time on their respective concentration (see Table S4, Supporting Information). Dunnett's tests showed that the concentrations of linalool in U3 and U4 were significantly higher than those in the control samples U1 ($p < 0.01$). Similarly, the concentrations of eugenol were significantly higher in U3 and U4 than in the control samples U1 ($p < 0.05$). For 1,8-cineole, the concentrations of U4 were significantly higher than in the control samples U1 ($p < 0.05$). For cuminaldehyde, a comparison between the concentrations in U4 and U1 failed to reach significance ($p = 0.097$). The differences in the concentrations of the other target compounds were not statistically significant ($p > 0.1$).

#### *3.1.2. Odor Activity Values*

For the respective highest concentrations per urine set, the average OAV for eugenol was 4.5 (range, considering the respective highest odorant concentrations of the study participants: 0.2–18), for linalool 2.4 (range: 0.3–4.9) and for 1,8-cineole 0.3 (range:

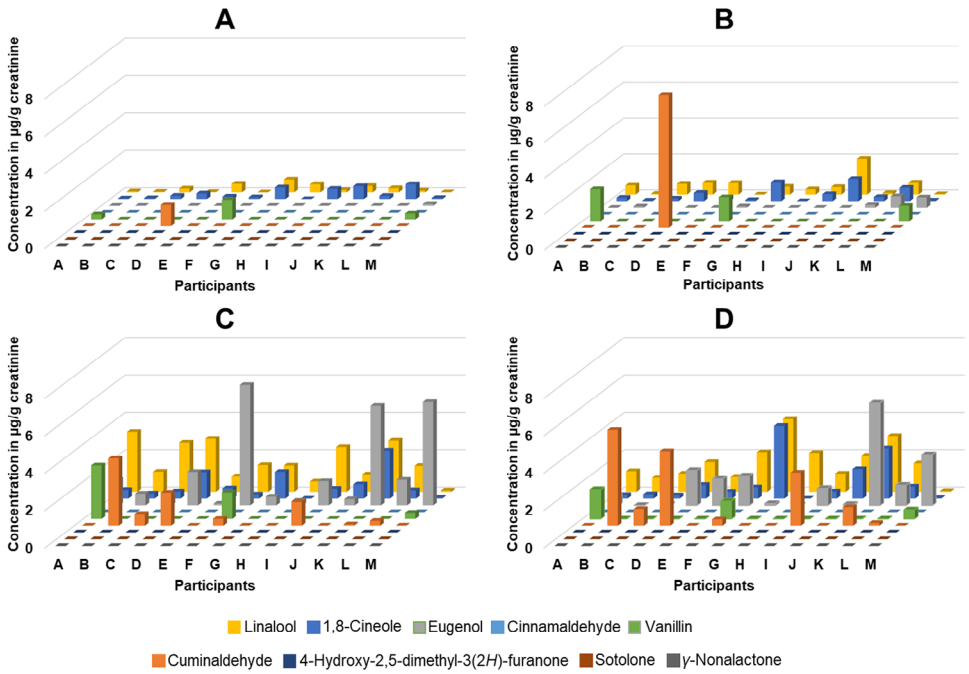


**Figure 1.** Overview of the concentration (in µg $g^{-1}$ creatinine) profiles of the nine target odorants in urine samples obtained from the 13 participants before consumption of the curry dish (A) and 0.5 h (B), 1.5 h (C), and 2.5 h (D) after consumption of the curry dish. The participants A–M correspond to participants A–M in Debong et al. (2021).

0–1.0). The maximum OAVs of cinnamaldehyde, vanillin, cuminaldehyde, HDF, sotolone, and γ-nonalactone were below 0.1, based on either their highest detected value or their LOD.

### *3.1.3. Comparison with the Aroma Transfer into Milk*

The concentrations of the target odorants in milk have been published previously (Debong et al., 2021). Similar to urine, the concentrations of the substances cinnamaldehyde, vanillin, HDF, sotolone, and γ-nonalactone were above the LOD in none or only a few samples. For milk, this was also the case for cuminaldehyde and eugenol. In contrast, a transfer of these two substances into urine became evident from the increased concentrations in the urine samples of seven and 13 participants, respectively. Finally, for linalool and 1,8-cineole, a transfer linked to curry consumption became evident for milk and urine, despite quantifiable concentrations of these two substances being already detected in the control samples of both milk and urine.

To compare the transfer of odorants into milk and urine in terms of transition velocity, **Figure 3** presents the extrapolated courses based on the average concentrations of linalool and 1,8-cineole, for which a relevant transfer into both urine and milk occurred. However, it must be stated that the actual courses might differ for the points in time between sampling points.

In the case of the milk samples, linalool and 1,8-cineole rose to their respective maximum concentration in the first sample which was donated 1 h after consumption of the curry dish. In contrast, for the transition into urine, the maximum concentrations were reached in the third sample (donated 1.5 h after the intervention) for 1,8-cineole and in the fourth sample (donated 2.5 h after the intervention) for linalool. Donations of milk and urine samples alternated, with 30 min between each donation, to facilitate participation in the study. This needs to be considered when comparing the temporal course of excretion into milk and urine.

Furthermore, the excretion rates into urine and milk were determined. The average cumulated absolute amount was 62 ng ($1.8 \times 10^{-4}$%) for linalool, 32 ng ($8.1 \times 10^{-3}$%) for 1,8-cineole, 35 ng ($1.6 \times 10^{-4}$ %) for cuminaldehyde, and 85 ng ($8.1 \times 10^{-3}$ %) for eugenol in the urine samples. For the transition into milk, those of linalool and 1,8-cineole in the milk samples after the intervention were 134 and 104 ng resulting in average transition rates of $3.9 \times 10^{-4}$% and $2.6 \times 10^{-2}$%, respectively.

## 3.2. Experiment 2: Determination of Conjugated Target Compounds

In Experiment 2, glucuronidase assays were conducted to determine the number of conjugated aroma compounds in urine samples obtained from non-lactating women before and after ingestion of the curry dish. The results are compiled in Table S5, Supporting Information and shown in **Figure 4**. Unfortunately, for 1,8-cineole, HDF, sotolone, and γ-nonalactone, no reliable data could be obtained due to a complication during the quantification experiments. The experiments could, however, not be repeated concisely comprising the same subjects and experimental framework, as comparability would have been compromised. Therefore, only the data for linalool, eugenol, cinnamaldehyde, vanillin, and cuminaldehyde are reported.

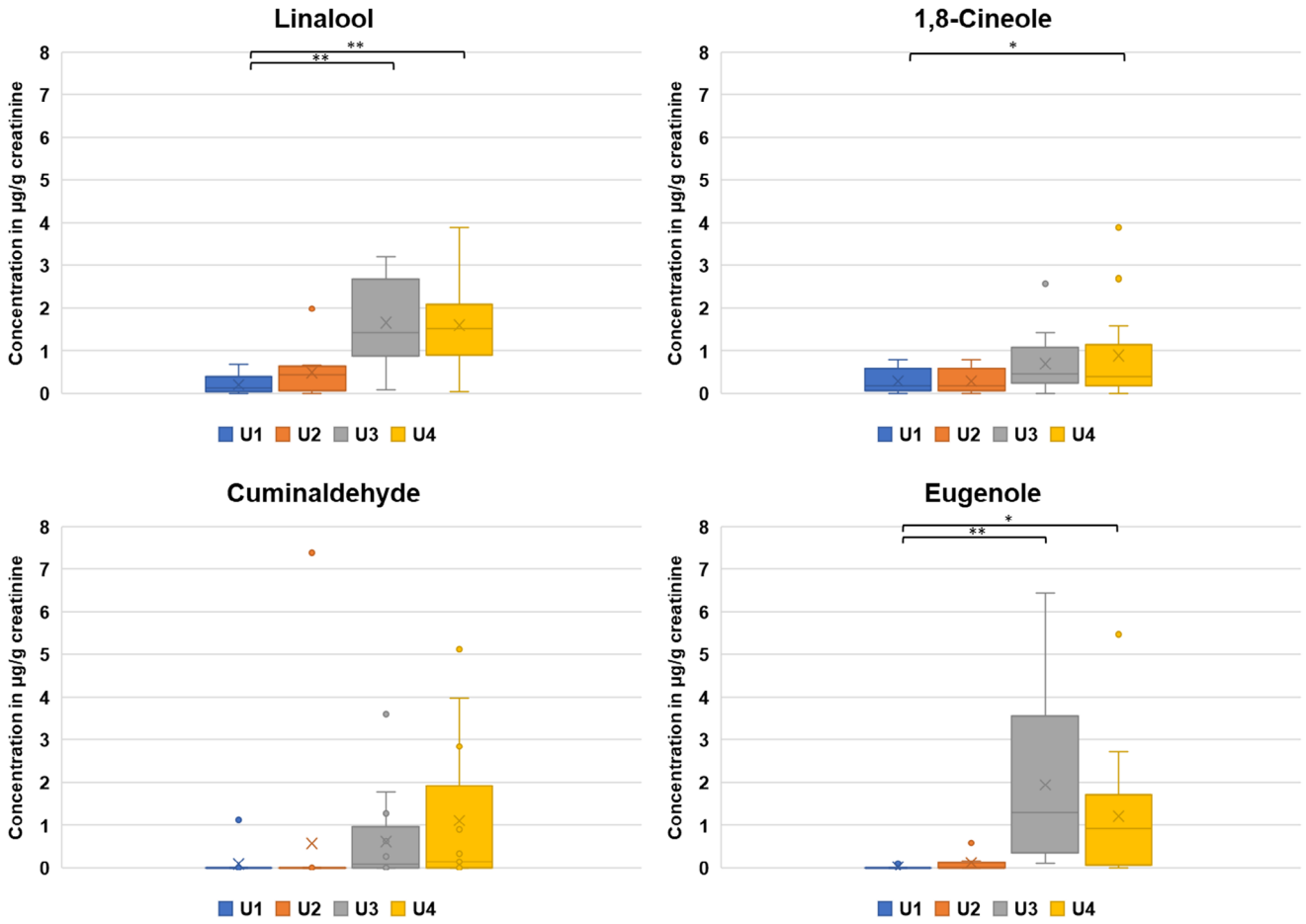


**Figure 2.** Concentration (in µg $g^{-1}$ creatinine) courses of linalool, 1,8-cineole, cuminaldehyde, and eugenol in urine samples, presented as boxplots indicating the median (middle line), the average (cross), the second and third quartile (box), the maxima, and minima in the range of up to the 1.5-fold length of the interquartile distance (whiskers) as well as the overall maxima and minima (dots). The urine samples were obtained before (U1) or after consumption of the curry dish (U2, U3, U4: 0.5, 1.5, and 2.5 h after consumption). * Significant with $p < 0.05$ (Dunnett's test); ** significant with $p < 0.01$ (Dunnett's test).

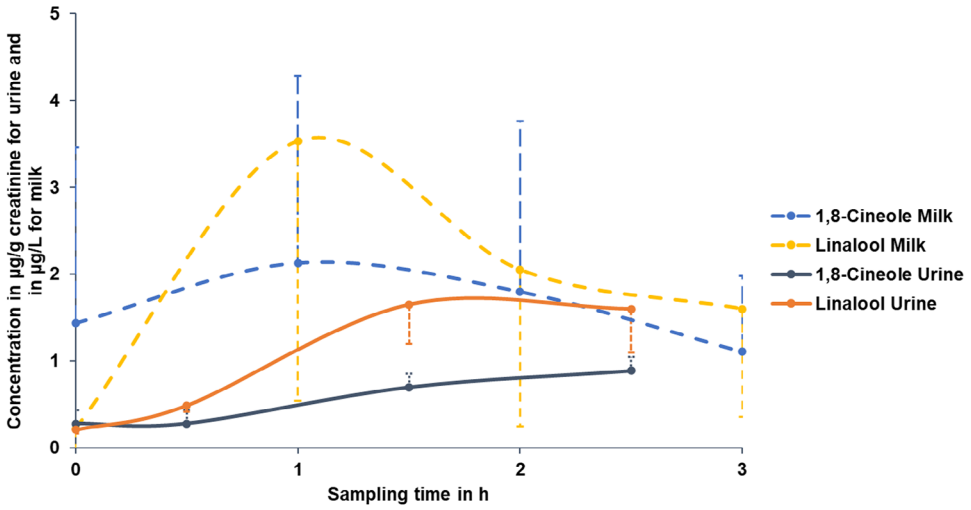


**Figure 3.** Extrapolated courses based on average concentrations of linalool (yellow) and 1,8-cineole (blue) in the samples before (sampling time 0 h) and after (sampling time 0.5–3 h) the consumption of the curry dish. Concentrations of the milk samples (dashed lines) are displayed in µg $L^{-1}$ and concentrations of the urine samples (solid lines) in µg $g^{-1}$ creatinine. Displayed are also the standard deviations in positive direction for 1,8-cineole and in negative direction for linalool.

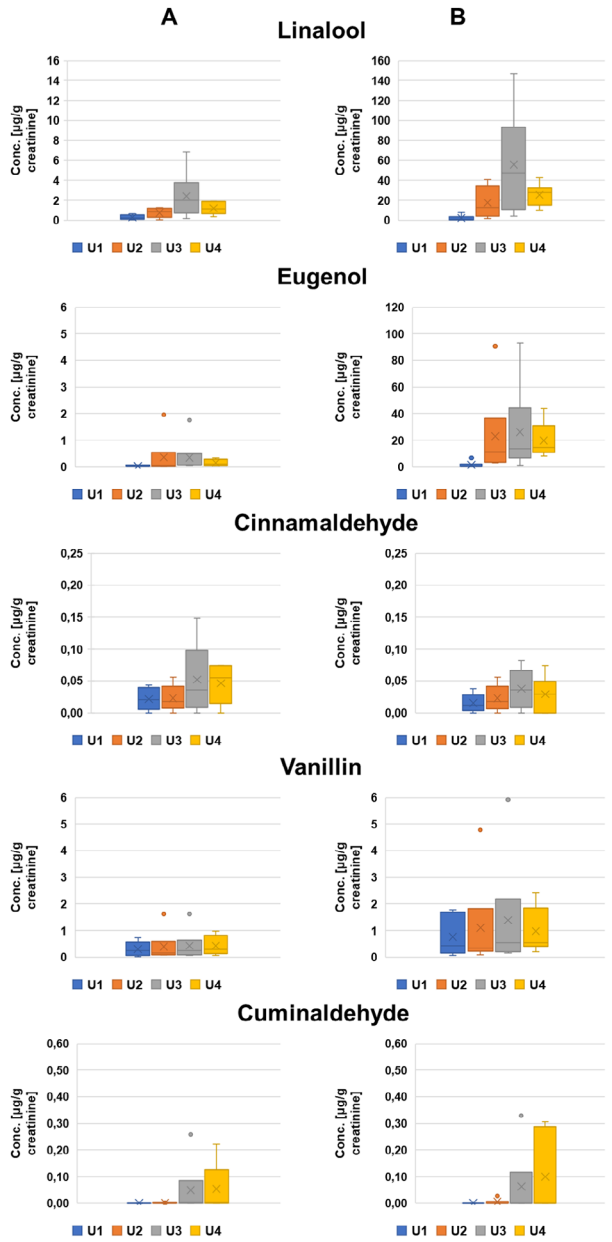


**Figure 4.** Boxplots of the concentration (in µg $g^{-1}$ creatinine) courses of linalool, eugenol, cinnamaldehyde, vanillin, and cuminaldehyde in urine samples obtained from six non-lactating women before (U1) and 0.5, 1.5, 2.5 h after (U2-4) the ingestion of a curry dish. Column A represents the determined concentrations without enzymatic treatment, while column B represents the concentrations with the treatment of glucuronidase. Observe different *y*-axis scaling for linalool and eugenol. The boxes represent the second and third quartile of the concentrations, and the whiskers two standard abbreviations. The *x* shows the average concentration while the middle line is the median concentration.

Prior to the intervention, the linalool concentration of the untreated samples ranged from 0.2 to 6.2 µg $g^{-1}$ creatinine (average: 2.9 µg $g^{-1}$ creatinine), and the concentration in the treated samples from 1.1 to 75 µg $g^{-1}$ creatinine (average: 20 µg $g^{-1}$ creatinine). After the intervention, the linalool concentration rose up to 6.8 µg $g^{-1}$ creatinine for the untreated and up to 147 µg $g^{-1}$ creatinine for the treated samples. The initial eugenol concentration in the untreated samples was detected in an area from below LOD to 0.5 µg $g^{-1}$ creatinine (average 0.2 µg $g^{-1}$ creatinine) and from 1.0 to 68 µg $g^{-1}$ creatinine for the treated samples (average 13 µg $g^{-1}$ creatinine). After the intervention, the concentration in the untreated samples rose up to 20 µg $g^{-1}$ creatinine and up to 933 µg $g^{-1}$ creatinine for the treated samples.

In case of cinnamaldehyde, the concentration before intervention in the untreated and the treated samples ranged from below the LOD to 0.4 µg $g^{-1}$ creatinine (average 0.2 µg $g^{-1}$ creatinine). After the intervention, the concentration in the untreated samples rose up to 1.5 µg $g^{-1}$ creatinine and up to 0.8 µg $g^{-1}$ creatinine for the treated samples. The vanillin concentration before intervention in the untreated samples ranged from 0.2 to 7.3 µg $g^{-1}$ creatinine (average 3.0 µg $g^{-1}$ creatinine) and from 0.7 to 17.7 µg $g^{-1}$ creatinine (average 7.5 µg $g^{-1}$ creatinine). After the intervention, the concentration in the untreated samples rose up to 16.2 µg $g^{-1}$ creatinine and up to 59.2 µg $g^{-1}$ creatinine for the treated samples. The initial cuminaldehyde concentration in the treated and the untreated samples remained below LOD in all samples. After the intervention, the concentration in the untreated samples rose up to 9.3 µg $g^{-1}$ creatinine and up to 3.3 µg $g^{-1}$ creatinine for the treated samples.

To evaluate the influence of the glucuronidase assay on the determined concentrations of the target compounds, statistical tests were performed. The concentrations of linalool, eugenol, and vanillin were significantly higher when treated with glucuronidase than without treatment ($p < 0.05$, Wilcoxon's test). For cinnamaldehyde and cuminaldehyde, the concentrations were not significantly different ($p > 0.05$) between experiments with and without glucuronidase treatment. Regarding a time-dependent transfer of glucuronides into urine, an ANOVA revealed a significant impact of sampling time on the concentration of linalool in the treated samples (Table S6, Supporting Information). The linalool concentration in the control sample was significantly lower than the concentration in the treated urine sample obtained 1.5 h after ingestion of the curry dish ($p < 0.05$, Dunnett's test). For the other target compounds, no statistically significant increase occurred in the treated samples ($p > 0.05$).

## 4. Discussion

In this study, the possible transfer of nine aroma compounds from a standardized curry dish into urine was determined. In the first experiment, the target substances were quantified in urine samples from lactating women who also donated milk samples.[4] The concentrations of linalool, 1,8-cineole, and eugenol in urine increased significantly around 1.5–2.5 h after consumption of the curry dish. A direct transfer of these substances into urine in their free form was shown in previous studies for eugenol[13] and 1,8-cineole,[14] however, to the best of our knowledge, not yet for linalool. For cuminaldehyde and vanillin, no statistically significant concentration difference could be determined. However, the concentration of cuminaldehyde increased from values mostly below the LOD before curry consumption to an average of 1.1 µg $g^{-1}$ creatinine at 2.5 h after intervention, so statistical significance might be reached in a larger sample size. Similarly, this could be the case for vanillin. On the other hand, a trans-

fer of cinnamaldehyde, γ-nonalactone, sotolone, and HDF into the urine samples did not become evident. In general, the transfer patterns differed between the participants. For some of them, eugenol was the highest concentrated odorant in their urine samples, while for others, it was linalool or cuminaldehyde. Furthermore, a closer look at the transfer of the target odorants into urine showed that the excreted amount was not directly proportional to the total amount of intake. The absolute cumulated amounts of transferred linalool and eugenol, for instance, were in a similar range, despite a 30 times higher ingestion of linalool. In contrast, the excretion rate of cuminaldehyde was in similar proportion to that of linalool, with $1.6 \times 10^{-4}$% and $1.8 \times 10^{-4}$%, respectively. Moreover, cinnamaldehyde could not be detected in the urine samples despite a three times higher concentration in the curry dish than that of eugenol (see Section 2.3). The transition rate of 1,8-cineole was equivalent to that of eugenol ($8.1 \times 10^{-3}$%). Vanillin was too rarely detected in the samples to draw any conclusion. Finally, for some of the substances, the highest concentrations were detected 2.5 h instead of 0.5 or 1.5 h after intervention. In these cases, the maximum of the transition may occur in a delayed manner, potentially also after the end of the sampling period. Inter-individual and substance-specific variations in metabolization and excretion patterns of aroma compounds as observed here have already been described previously[8a,14,15] and are the consequence of a person's individual uptake, metabolization, and excretion processes as well as a substance's metabolic pathway.[16] Amongst others these individual differences could derive from a person's individual microbiome[17] as well as enzymatic differences in the individual phase II metabolism.[18] Potential additional excretion pathways include the formation of phase I metabolites and their phase II metabolism conjugates which would not have been detected within this study. Furthermore, the excretion via the breath as, e.g., shown for 1,8-cineole[19] could be an alternative excretion pathway next to that via skin and skin glands or feces. In addition, upon ingestion of a complex mixture of food constituents, as was the case here, competitive or inhibitory effects within metabolic pathways as well as matrix influences on uptake and biotransformation rates are to be expected.

Since the participants of the first experiment also donated milk samples[4] a direct comparison of the aroma transfer into milk and urine was possible. Linalool and 1,8-cineole were transferred into both milk and urine. On the other hand, an increase in eugenol and cuminaldehyde concentrations was detected in urine but not in the milk samples. Accordingly, the transition of aroma compounds into urine appears to be relevant for a higher number of odorants, even though the transfer profiles differed between the individual participants in both specimens. This may be explained by an evolutionarily more restricted transfer of substances into milk whereas urine serves as a classical excretion specimen. A more detailed comparison of the data obtained for the individual participants further revealed that i) a transition of vanillin into milk and urine, respectively, was detected for the same three mothers, and ii) participant D had especially high cuminaldehyde concentrations in urine as well as in the milk samples. These results could either be explained by a different metabolism of the respective participants or a lack of compliance during the wash-out phase. However, no exceptional consumption of vanillin or cuminaldehyde-containing food was found in the nutritional diaries of these participants. Therefore, these results may indeed point towards a different metabolization and excretion of these compounds in the individual participants.

Considering the temporal course of aroma transfer, the results indicate that the transition into milk is faster than into urine. Nevertheless, the offset between the sampling of milk and urine prevents a final conclusion in this respect. We are not aware of any previous reports comparing the temporal profile of aroma transfer into urine and milk in the same participants. Previous studies were run on urine and milk samples obtained from different participants[7b,14,15] or focused on odorant metabolites,[20] the temporal excretion of which might not be related to the one of the parent compounds. If the here observed temporal offset is confirmed in future studies, different explanations could be given. A first potential driver for a temporal excretion difference might be the shorter physical distance between the points uptake of the odorants and the breast in comparison to the bladder which would be especially relevant if the transfer mechanism is mainly diffusion. Another potential driver could be a larger storage offset by the bladder itself compared to the breast. Finally, there might also be a tendency of more hydrophile substances rather being extrected via urine than milk while these substances are less likely able to permeate through cell structures but rather being transported through the whole body within the blood stream.

Analogously to our previous study on milk,[4] OAVs were determined to estimate whether the aroma transfer into urine might be olfactorily perceivable. For linalool as well as for eugenol an OAV > 1 was found in most samples after the intervention, which is why it is assumed that their transition could also be perceived by the human nose in urine. For 1,8-cineole an OAV of nearly 1 was calculated for two participants, thus in certain cases, a transfer of 1,8-cineole might be perceivable while for the other target odorants an influence on the overall odor of urine appears unlikely. Especially for urine, however, such conclusions need to be confirmed by experimental data because previous investigations of our group have shown that the odor of urine can mask an aroma transfer from the human diet (unpublished data) while the odor thresholds in this study were determined in water. Moreover, it is well known that the OAV concept does not account for interactions of odorants at the perceptual level and the impact of aroma release in a complex matrix.[12,21]

Overall, an important metabolic pathway for the excretion of odorants into liquid media like blood, saliva or urine is glucuronidation as part of the phase II metabolism. To evaluate the role of this pathway in the excretion of the target compounds into urine, glucuronidase assays were performed in Experiment 2 with urine samples obtained from six non-lactating women. Quantitative data were obtained for linalool, eugenol, cinnamaldehyde, vanillin, and cuminaldehyde. The results obtained from the untreated samples confirmed the outcome of Experiment 1: linalool, eugenol, and cuminaldehyde, but not vanillin, were transferred into urine in their unconjugated form. In contrast to Experiment 1, however, cinnamaldehyde was detected. Possibly, uptake and/or metabolic routes of cinnamaldehyde differ between lactating and non-lactating women. However, this would have to be studied within a further study. Physiological changes due to lactation are known in relation to calcium and zinc excretion[22] but also to other aspects of metabolism.[23] Taking into account the concentrations in the treated samples, it

became evident that for eugenol, linalool, and vanillin most of the excretion occurred via phase II metabolites as their concentrations rose on average 77-, 23-, and 3-fold, respectively, after deconjugation. Although these results indicate that the degree of phase II metabolization differs between these substances, they do not fully explain the substance-specific differences regarding their excretion rate in relation to the dosage of the substances in the curry dish. Therefore, further differences in metabolization as well as in the routes of excretion must exist and account for the different transfer rates. Our results support previous reports on the excretion of linalool, eugenol, and vanillin as glucuronides. For eugenol, Fischer et al.[13] showed in an intervention study that, on average, 95% of ingested eugenol (dosage: 150 mg) was excreted via urine within 24 h, of which 55% was excreted as eugenol-glucuronide, with the remaining proportion being excreted as conjugates of phase I metabolites. For linalool, Aprotosaoaie et al.[24] stated in their review that in rats, urine is the main excretion route as well, though with a lower proportion of 60%. Furthermore, the metabolite spectrum for linalool was shown to be more diverse with linalool-glucuronide being amongst seven further possible metabolites. The here observed significant increase in vanillin concentration after the glucuronidase treatment further confirms the postulate of Wagenstaller et al.,[8a] stating that glucuronidation of vanillin is probable due to its structure.

For cuminaldehyde and cinnamaldehyde, no significant differences were found between treated and untreated samples, which is why it can be assumed that no phase II conjugation of the parent substance and excretion into urine occurred for these substances. A further explanation lies in the mechanism of glucuronic acid conjugation and deconjugation. As the glucuronidase cleaves the ether connection of the glucuronide again into the glucuronic acid and a hydroxy-group of the respective odorant, only parent compounds with hydroxy-groups can be reconstituted by the glucuronidase. Therefore, no release for cinnam- and cuminaldehyde by the glucuronidase assay could be expected. For both substances or aldehydes in general, an intense phase-I metabolism is known, transforming them to their respective acids and alcohols,[25] after which glucuronidation is a plausible next step. However, corresponding metabolites were not detected in our study.

#### 4.1. Summary and Outlook

A significant time-dependent transition of the odorants linalool, 1,8-cineole, and eugenol into the urine of nursing mothers was demonstrated after the ingestion of a standardized curry dish. This transition accompanied (or preceded) the previously reported excretion of linalool and 1,8-cineole into human milk. For linalool and eugenol, the average OAVs in urine were >1. Therefore, their transfer might be olfactorily perceivable. According to the OAVs obtained for 1,8-cineole, this compound might be perceived only in some cases. For the remaining six target odorants, neither a significant transfer was detected nor were the average OAVs after the intervention higher than 1. By comparing the estimated excretion rates of the transferred odorants into urine and milk, it appears that transition occurs in similar ratios for free linalool and 1,8-cineole, but qualitative differences exist regarding which odorants were found to be transferred. Finally, an additional study with non-lactating women allowed us to confirm for a selection of target compounds (linalool, eugenol, and vanillin) that the direct excretion of odorants into urine is quantitatively less important than the excretion of the corresponding glucuronides.

A question that remains, and which should be targeted in future studies is whether odorant glucuronides occur in human milk as it was shown for certain drugs like propanolol.[26] Furthermore, phase-I metabolites like the alcohols and acids of cinnam- and cuminaldehyde, as well as their conjugates, should be investigated. Finally, the so far obtained results suggest that in line with previously published results for milk[4a] part of the odorants, notably cuminaldehyde, has not been completely excreted within the duration of the study. Thus, longer sampling periods should be applied in future studies to not only ensure the coverage of the complete substance transition but also to determine at which point the odorant concentrations go down to their base levels. To facilitate an even more comprehensive insight into the fate of odorants and their metabolites further excretion routes like feces or breath may be advisable to be also investigated.

## Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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## Conflict of Interest

The authors declare no conflict of interest.

## Author Contributions

M.D. did the quantitative and qualitative analysis, untargeted data acquisition, data analysis, and interpretation, supervised the glucuronidase experiments, human intervention study, and wrote the manuscript. K.N. contributed to data interpretation and the manuscript. Y.Y. did the glucuronidase experiments. R.L. conceptualized and supervised the project and contributed to data interpretation and the manuscript. T.H. conceptualized and supervised the project and contributed to data interpretation and the manuscript. A.B. conceptualized and supervised the project and contributed to data interpretation and the manuscript. H.L. realized the human intervention study, conceptualized and supervised the project, and contributed to data interpretation and the manuscript.

## Data Availability Statement

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

## Keywords

aroma transition, gas chromatography-mass spectrometry/olfactometry, maternal diet, milk, phase II-metabolism

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[1] a) D. W. Guthrie, S. Humphreys, *Diabetes Educ.* **1988**, *14*, 521; b) M. J. Enk, G. Oliveira e Silva, N. B. Rodrigues, *PLoS One* **2012**, *7*, e38947; c) U. Y. Schaff, G. J. Sommer, *Clin. Chem.* **2011**, *57*, 753; d) K. Skogstrand, C. K. Ekelund, P. B. Thorsen, I. Vogel, B. Jacobsson, B. Nørgaard-Pedersen, D. M. Hougaard, *J. Immunol.* **2008**, *336*, 78.

[2] a) C. M. Berlin Jr, H. M. Denson, C. Daniel, R. M. Ward, *Pediatrics* **1984**, *73*, 59; b) L. Scheffler, Y. Sauermann, A. Heinlein, C. Sharapa, A. Buettner, *Metabolites* **2016**, *6*, 43; c) J. A. Mennella, P. L. Garcia-Gomez, *Alcohol* **2001**, *25*, 153.

[3] a) J. A. Mennella, G. K. Beauchamp, *Infant Behav. Dev.* **1996**, *19*, 13; b) A. K. Ventura, *Ann. Nutr. Metab.* **2017**, *70*, 8.

[4] a) M. W. Debong, K. N'Diaye, D. Owsienko, D. Schöberl, T. Ammar, R. Lang, A. Buettner, T. Hofmann, H. M. Loos, *Mol. Nutr. Food Res.* **2021**, *65*, 2100507; b) K. N´Diaye, M. Debong, J. Behr, S. Dirndorfer, T. Duggan, A. Beusch, V. Schlagbauer, C. Dawid, H. M. Loos, A. Buettner, R. Lang, T. Hofmann, *Mol. Nutr. Food Res.* **2021**, *65*, 2100508.

[5] A. Mobasheri, R. Barrett-Jolley, *J. Mammary Gland Biol. Neoplasia* **2014**, *19*, 91.

[6] E. P. Widmaier, H. Raff, K. T. Strang, in *Vander's Human Physiology - The Mechanisms of Body Function* (Eds: F. S. K. A. Peterson, M. Watnick), The McGraw-Hill Companies, Boston, Mass., USA **2001**, p. 505.

[7] a) F. Kirsch, A. Buettner, *Metabolites* **2013**, *3*, 47; b) L. Scheffler, Y. Sauermann, G. Zeh, K. Hauf, A. Heinlein, C. Sharapa, A. Buettner, *Metabolites* **2016**, *6*, 18.

[8] a) M. Wagenstaller, A. Buettner, *Metabolites* **2013**, *3*, 637; b) G. Frigerio, R. Mercadante, E. Polledri, P. Missineo, L. Campo, S. Fustinoni, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2019**, *1117*, 66; c) A. K. Orlovius, S. Guddat, M. K. Parr, M. Kohler, M. Gütschow, M. Thevis, W. Schänzer, *Drug Test. Anal.* **2009**, *1*, 568.

[9] W. Engel, W. Bahr, P. Schieberle, *Eur. Food Res. Technol.* **1999**, *209*, 237.

[10] C. C. Sweeley, W. H. Elliott, I. Fries, R. Ryhage, *Anal. Chem.* **1966**, *38*, 1549.

[11] E. Kováts, *Helv. Chim. Acta* **1958**, *41*, 1915.

[12] M. Rothe, B. Thomas, *Z Lebensm Unters Forsch* **1963**, *119*, 302.

[13] I. U. Fischer, G. E. Von Unruh, H. J. Dengler, *Xenobiotica* **1990**, *20*, 209.

[14] M. Schaffarczyk, T. S. Balaban, M. Rychlik, A. Buettner, *ChemPlusChem* **2013**, *78*, 77.

[15] F. Kirsch, K. Horst, W. Röhrig, M. Rychlik, A. Buettner, *Metabolomics* **2013**, *9*, 483.

[16] N. Kornbausch, M. W. Debong, A. Buettner, J.-M. Heydel, H. M. Loos, *Angew. Chem., Int. Ed.* **2022**, *61*, e202202866.

[17] A. Visconti, C. I. L. Roy, F. Rosa, N. Rossi, N. Rossi, T. C. Martin, T. C. Martin, R. P. Mohney, W. Li, E. d. Rinaldis, J. T. Bell, J. C. Venter, K. E. Nelson, T. D. Spector, M. Falchi, *Nat. Commun.* **2019**, *10*, 4505.

[18] A. H. Nishioka, E. d. C. Tobaruela, L. N. Fraga, F. A. Tomás-Barberán, F. M. Lajolo, N. M. A. Hassimotto, *Nutrients* **2021**, *13*, 473.

[19] J. Beauchamp, F. Kirsch, A. Buettner, *J Breath Res* **2010**, *4*, 026006.

[20] L. Scheffler, C. Sharapa, T. Amar, A. Buettner, *Front. Chem.* **2018**, *6*, 410.

[21] a) S. S. Stevens, *Sci. Am.* **1960**, *48*, 226; b) M. Czerny, M. Christlbauer, M. Christlbauer, A. Fischer, M. Granvogl, M. Hammer, C. Hartl, N. M. Hernandez, P. Schieberle, *Eur. Food Res. Technol.* **2008**, *228*, 265.

[22] a) C. J. Klein, P. B. Moser-Veillon, L. W. Douglass, K. A. Ruben, O. Trocki, *Am. J. Clin. Nutr.* **1995**, *61*, 779; b) V. J. Taylor, *Mol. Cell* **2023**, *559*, 111797.

[23] M. Martinez, S. Modric, *J. Vet. Pharmacol. Ther.* **2010**, *33*, 213.

[24] A. C. Aprotosoaie, M. Hăncianu, I.-I. Costache, A. Miron, *Flavour Fragr. J.* **2014**, *29*, 193.

[25] a) T. Ishida, M. Toyota, Y. Asakawa, *Xenobiotica* **1989**, *19*, 843; b) A. Buettner, *J. Agr. Food Chem.* **2002**, *50*, 7105.

[26] M. T. Smith, I. Livingstone, W. D. Hooper, M. J. Eadie, E. J. Triggs, *Ther. Drug Monit.* **1983**, *5*, 87.