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Dietary Piperine is Transferred into the Milk of Nursing Mothers

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Introduction: The diet of breastfeeding mothers could bring nurslings into contact with flavor compounds putatively contributing to early sensory programming of the infant. The study investigates whether tastants from a customary curry dish consumed by mothers are detectable in their milk afterwards and can be perceived by the infant.

Methods and Results: Sensory evaluation identifies pungency as the dominating taste impression of the curry dish. Its ingredients of chili, pepper, and ginger suggest the flavor compounds capsaicin, piperine, and 6-gingerol as analytical targets. Breastfeeding mothers are recruited for an intervention trial involving the consumption of the curry dish and subsequent collection of milk samples for flavor compound analysis. Targeted and untargeted mass spectrometric (MS)- investigations identify exclusively piperine as an intervention-derived compound in human milk. However, concentrations are below the human taste threshold.

Conclusion: Piperine from pepper-containing foods transfers into the mother's milk within 1 h and is delivered to the nursling. Concentrations of 50 and 200 nM of piperine are 70–350 times below the human taste threshold, but TRPV1 (Transient Receptor Potential Vanilloid-1 ion channel) desensitization through frequent exposure to sub-taste-threshold concentrations could contribute to an increased tolerance at a later age.

1. Introduction

A balanced diet supplies sufficient amounts of macro- and micronutrients and is mandatory to secure complete functionality of the adult body and maintain good health. Beyond the pure intake of energy and building blocks, sensorial attractiveness, and individual preferences strongly affect food choice, as eating and drinking is a daily procedure with fundamental hedonic aspects. Beside taste preferences adopted in later life, such as the bitter taste of coffee, some preferences originate from sensory programming in the first months of life through exposure to human milk (hereafter referred to as milk).[1,2] For several odor-active compounds, a dietary transfer into milk has been demonstrated on a molecular level[3–5] and is the basis of such early flavor learning. However, research in this area has also shown that aroma transfer cannot be assumed to be relevant for each dietary intake,[6,7] and biotransformation has a considerable impact on the nature of compounds transferred into milk.[4,8,9] Compared with odor-active substances, much less attention has been paid to the potential transfer of dietary constituents, which are taste-active or contribute to food flavor though other chemosensory properties. These compounds have incidentally been used in intervention studies with breastfeeding mothers, such as investigations of bitter tasting caffeine, which is known for its stimulating activity and can be transferred into milk.[10] Similarly, the

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taste properties of milk have rarely been assessed.[11] Only recently, Mastorakou et al.[12] evaluated the relationship between the bitter taste of milk and the bitter content of the mothers' diets. For other taste impressions, only sketchy evidence exists. Therefore, as mentioned in Debong et al. (this issue), we conducted an intervention study that allowed us to characterize the transfer of flavor compounds from a customary meal into milk to further our understanding of how comprehensively the flavor of a customary dish might be transferred to milk. The results of this study are presented in two publications: the current work focusing on taste-active compounds and non-volatile metabolites, and Debong et al. (this issue) reporting on the results obtained for volatile odor-active compounds.

A curry dish was selected for the investigation of flavor-active compounds. Curry dishes are frequently consumed in many cultures globally and bear different taste impressions. Typical curry dishes can comprise bitter tastants from cinnamon, sweet saccharides from coconut milk, umami tastants from protein sources, salt, acidic ingredients, and other chemosensorially active compounds, such as astringent polyphenols, or pungent substances, such as capsaicin and piperine from chili and pepper, respectively. Sensory evaluation of the curry powder and the curry dish in combination with targeted and untargeted mass spectrometric investigations of the curry powder, curry dish, and milk samples were used to characterize the temporal and quantitative course of taste transfer into milk. We aimed to estimate the sensory relevance of the detected flavor transfer to nurslings using additional threshold determinations of the respective tastants.

2. Experimental Section

2.1. Chemicals and Materials

MS-grade solvents used for liquid chromatography-mass spectrometry (LC-MS) measurements were purchased from Honeywell Burdick & Jackson (Seelze, Germany). Ultrapure water was prepared as solvent for LC-MS measurements using a Milli-Q apparatus (Millipore, Schwalbach, Germany). Formic acid was obtained from Merck (Darmstadt, Germany) to adjust the pH of the LC-MS solvents. Capsaicin, curcumin, dihydrocapsaicin, 6-gingerole, piperine (food grade quality), nonivamide, 6-shogaol, and standard compounds for taste analysis (ethanol, caffeine, sodium chloride, sucrose, mono sodium glutamate, citric acid, tannic acid, and rutin) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Cf. supporting information for the synthetic procedures of the internal standards d_{10}-piperine and d_3-nonivamide. Cow's milk (3.5% fat, homogenized, pasteurized), follow-on formula "Humana Folgemilch 2" (Humana GmbH, Herford, Germany), and water for sensory evaluation (Evian, Danone S.A., Paris, France) were purchased from local supermarkets. The ingredients of the selected curry powder for the intervention study are in the supporting information and Debong et al. (this issue).

2.2. Sensory Characterization

2.2.1. Consensus Taste Profile of Curry Powder and Curry Dish

A consensus taste profile of the selected curry powder and dish in accordance with DIN EN ISO 13 299 was performed. First, the trained panel (n = 5, m/w 3/2, mean age 29 years) was provided with characteristic reference solutions, and they agreed on a common terminology and definitions for each taste descriptor and its intensity: (sour was 20 mM citric acid, sweet was 29 mM sucrose, bitter was 15 mM caffeine, salty was 200 mM NaCl, umami was 20 mM mono sodium glutamate, rough-astringent was 6 µM tannic acid, velvet-astringent was 2 µM rutin, and pungent was 0.035 µmol of piperine per filter paper stripe. A defined scale from 0 (complete absence) to 3 (very intense) was set. The test persons individually evaluated the respective samples and recorded their results on an evaluation sheet. Individual assessments were collected by the panel manager, who led the subsequent discussion. All previously defined groups of characteristics and their intensities were described in the discussion, and, if necessary, the reference solutions were provided for the final determination of taste intensity. After reaching a consensus, final descriptive taste profiles were created for the curry powder, the curry sauce, and the entire dish: (sauce with rice).

2.2.2. Estimation of the Taste Threshold of Piperine in Human Milk

The human taste threshold of piperine was estimated using a triangle test-scenario.[13] Participants were non-smoking, healthy individuals trained in sensory analysis. A stock solution of piperine (food grade, 50 mg, 175 µmol) in ethanol (absolute, 5 mL) was prepared and aliquots spiked into follow-on formula (150 mL, prepared from tap water and formula powder according to the manufacturer's recipe: 4.6 g/150 mL water) to give a final concentration of 8.75 µM (n = 6 participants, m/w 3/3, age 25–30 years) and 17.5 µM (n = 7 participants, m/w 3/4, age 25–30 years). Each piperine-containing sample was blinded with two blank samples of follow-on formula each spiked with pure ethanol (75 µL 150 mL^{-1} sample). For each concentration level, one piperine-containing and two piperine-free samples were presented to the sensory panel. The samples were kept stirring on heating plates at 37°C to mimic the situation during nursing. Sensory evaluation was performed as sip and spit: evaluated solutions were spat out. Sensory water (Evian) with ethanol (1 %) was provided for mouth rinsing between concentrations. The collected data were analyzed according to.[13]

2.3. Human Intervention Study and Sample Collection

The study sampling protocol is presented in **Figure 1**. The study was designed in accordance with the Declaration of Helsinki, and the ethical committee of the Friedrich to Alexander-Universität Erlangen-Nürnberg approved the study protocol with registration number 24_16 B. Milk samples were collected using a mechanical breast pump (Medela Harmony, Medela AG, Baar, Switzerland). All samples were stored in brown glass at -80°C until analysis. The participants kept a nutrition diary and detailed their food intake in the two days prior to the intervention. Further details and dosage information are in the supporting information and Debong et al. (this issue). Two additional mothers agreed to collect small amounts (2 mL) of additional milk samples within 10 and 24 h after ingestion of the curry test meal, for analytical purposes. These samples were exclusively used for targeted analyses of pungent compounds.

2.4. Targeted Quantification of Pungent Compounds

2.4.1. Standard Solutions and Calibration

Methanolic stock solutions (3 mM, determined by qNMR[14]) of internal standards d_3-nonivamide and d_{10}-piperine were combined and diluted with methanol to 100 µM per compound used for the analysis of the curry spice mixture and to 100 nM per compound used for the analysis of milk samples by further 1:1000 dilution with methanol. Stock solutions of the analytes (6-gingerole, **1**; piperine, **2**; nonivamide, **3**; capsaicin, **4**; curcumine, **5**; dihydrocapsaicin, **6**; 6-shogaol, **7**) in methanol (1 mM) were combined and diluted with methanol to obtain a solution containing all

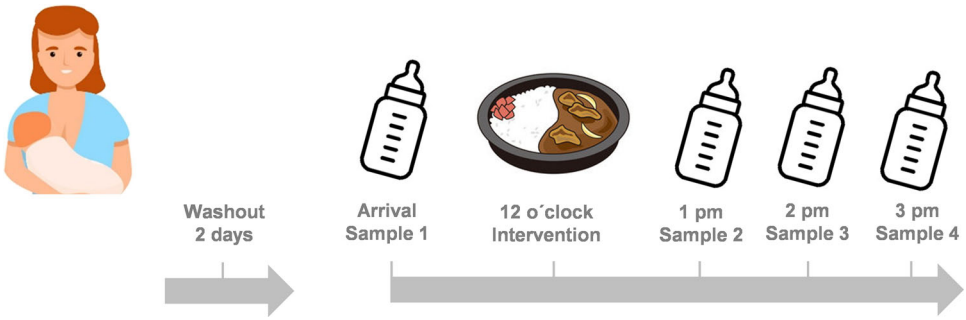

Figure 1. Overview of the intervention study scenario and the collected milk samples.

seven analytes at concentrations of 10 µM. This solution was diluted in ten 1+1-steps with methanol. Aliquots of these dilutions (100 µL) were spiked into aliquots of cow's milk (3.5% lipids, 3.5% protein, homogenized, pasteurized, 900 µL) to yield spiked matrix calibration standards with analyte concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.9, and 0.9 nM.

2.4.2. Quantification of Pungent Compounds in the Curry Spice Mixture and Curry Sauce

The homogenized spice sample (50–100 mg) was weighed into an Eppendorf cap, mixed with the internal standard solution (100 µM, 1 mL), and incubated in an ultrasonic bath at ambient temperature for 1 h. Then, the suspension was centrifuged (12 000 rpm, 5 min, 4°C), and the supernatant was diluted at 1/500 with 50% aqueous methanol and pipetted into an autosampler vial. An aliquot was injected into the liquid chromatography with tandem mass spectrometry (LC-MS/MS) system (2 µL).

2.4.3. Quantification of Compounds in Milk

Spiked matrix calibration samples and authentic milk samples (100 µL) were mixed with the internal standard (100 nM, 100 µL), vortexed, and diluted with methanol/acetonitrile (1/1, v/v, 400 µL). Then, the suspension was centrifuged, and the supernatant was decanted into another Eppendorf cap and evaporated in a stream of nitrogen. The residue was suspended in methanol/acetonitrile (1/1, v/v, 50 µL), mixed with water (50 µL), centrifuged (12 000 rpm, 5 min, 4°C), and transferred to a 200 µL-insert in an autosampler vial. The matrix standards of 500, 62.5, and 7.8 nM (high, medium, and low, respectively) were processed in further replicates ($n = 3$) as quality control (QC) samples to assess precision and accuracy. Additionally, authentic samples (volunteer 7668) were analyzed in replicates to determine method performance (cf. Table S1, Supporting Information).

2.4.4. Instrumentation for Targeted Analysis of Intact Pungent Compounds

The LC-MS/MS system consisted of an Ultimate 3000 ultra high performance liquid chromatography (UHPLC; Dionex, Idstein, Germany) and an API 4000 QTRAP (AB Sciex, Darmstadt, Germany). For targeted analysis of intact pungent compounds in human milk, samples and standards were chromatographed at 50°C on an RP18 column (Kinetex, C18, 100 × 2.1 mm, 1.7 µm, Phenomenex, Aschaffenburg, Germany) with a flow of 400 µL min^{-1} and 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water (B) using gradient elution. Starting at 35% B, A was increased to 100% within 5 min under nonlinear conditions (curve 8) and kept isocratic for 2 min before re-establishing the initial conditions within 0.2 min and re-equilibration for 2.8 min. The injection volume was 1 µL. The autosampler kept the samples at 20°C. The source temperature (ESI^+) was 500°C, ion spray voltage +5.5 kV, curtain gas, nebulizer gas (N_2), and drying gas (air) were 35, 50, and 60 psi, respectively. The following Q1/Q3 pairs (quantifier marked w/asterisk) were recorded for 20 ms each (the declustering potential, collision energy, and cell exit potential are in parenthesis): 6-gingerol (**1**) 295/177*, 137, 94 (66, 13/31/71, 6/4/4), piperine (**2**) 286/201*,115, 135 (81, 27/65/35, 16/10/8), d_{10}-piperine (**d_{10}-2**) 296/201*, 115, 135 (91, 31/67/37, 4/8/10), nonivamide (**3**) 294/137*, 94, 122 (41, 21/69/55, 10/6/8), d_3-nonivamide (**d_3-3**) 297/137*, 94, 122 (61, 25/67/55, 10/6/8), capsaicin (**4**) 306/137*, 94, 122 (61, 21/71/57, 10/6/8), curcumin (**5**) 369/177*, 145, 285 (86, 31/45/23, 14/12/8), dihydrocapsaicin (**6**) 308/137*, 94, 66 (66, 23/73/89, 10/6/4), 6-shogaol (**7**) 277/137*, 177, 145 (61, 19/17/33, 12/4/12).

MS/MS data were analyzed in Multiquant (Sciex), calculations were done using Excel and further analysis in Graphpad Prism 9.0.0. Quantitative data were analyzed by univariate analysis of variance (ANOVA), followed by Dunnett´s post hoc test. Samples were matched across sampling timepoints and compared to the control sample ($t = 0$). A representative analysis of a milk sample is provided in **Figure S1** (Supporting Information).

2.5. Untargeted Metabolomic Profiling of Human Milk

2.5.1. Sample Preparation for Untargeted Profiling of Human Milk Samples

An aliquot (100 µL) of the milk samples was mixed with methanol/acetonitrile (1/1, v/v, 400 µL) for protein precipitation, vortexed, and subsequently centrifuged (12 000 rpm, 5 min, 4°C). The supernatant (250 µL) was transferred into another Eppendorf cap, the solvent was evaporated in a stream of nitrogen, and the residue was suspended in methanol/acetonitrile (1/1, v/v, 25 µL) and diluted with ultra-purified water (25 µL). Individual milk samples ($n = 13$, cf. **Table S1**, Supporting Information) were worked up in triplicates. A pooled control sample ("control," aliquots taken before consumption of the test meal) and a pooled treated sample ("treated," milk sample collected 3 h after consumption of the test meal) were prepared by pooling aliquots of the respective individual samples (10 µL). An aliquot of each pooled sample was processed as above. One quality control sample (QC sample) was prepared ("QC13") comprising aliquots (10 µL) from all individual samples ("control" and "treated") for a system conditioning check and another QC sample ("QC10") without subjects 6955, 7654, and 7668 (high piperine concentrations in the control samples) for TOF-MS drift normalization and alignment.

2.5.2. Instrumentation for Untargeted Profiling of Human Milk Samples

Untargeted metabolomic profiling using a Sciex TripleTOF 6600 mass spectrometer was conducted (Sciex, Darmstadt, Germany) connected to

a Shimadzu Nexera X2 system (Shimadzu, Kyoto, Japan) operating in the positive and negative electrospray ionization mode. Data acquisition and instrumental control were conducted using AnalystTF software (v 1.7.1, Sciex, Darmstadt). Chromatographic separation was using an RP18 LC column (Kinetex C18, 100 × 2.1 mm, 1.7 µm, Phenomenex, Aschaffenburg, Germany) with gradient elution using acidified water (0.1% formic acid, A) and acetonitrile (0.1% formic acid, B). Elution started with 2% B for 3 min. Then, B was increased to 100% within 5 min and kept isocratic for 7 min. The starting conditions were re-established within 1 min and kept for 5 min for equilibration.

For system conditioning, QC13 was repeatedly injected ($n = 10$). In the acquisition batch, samples were injected in a randomized order with blank and QC injections (QC10 and QC13) every 10th injection. The column oven was set to 40°C, and the injection volume was 3 µL. Data Independent Acquisition (DIA) was performed using a TOF-MS scan in high resolution mode for 100 ms in the m/z range from 50 to 1000 Da, followed by 20 variable Q1 isolation windows with an acquisition time of 90 ms each. The variable Q1 isolation windows were calculated using swathTUNER based on precursor m/z frequencies of the prerun data using Information Dependent Acquisition (IDA) from the QC run with an overlap of 1 Da: m/z 50–113.33, 112.33–176.66, 175.66–239.99, 238.99–03.32, 302.32–366.65, 365.65–429.98, 428.98–493.31, 492.31–556.64, 555.64–619.97, 618.97–683.3, 682.3–746.63, 745.63–809.96, 808.96–873.29, 872.29–936.62, 935.62–1000.[15]

2.5.3. Untargeted Data Processing

Peak picking of raw data files was conducted using MS-Dial software as detailed in the supporting information.[16] In accordance with suggested guidelines,[39] data quality was assessed by visual inspection of total ion counts, retention times stability and peak intensities of QCs across the acquisition batch (TICs, cf. **Figure S2**, Supporting Information), visual inspection of principal component analysis (PCA) regarding the clustering QCs and blanks in comparison to the total variance (cf. **Figure 3** and **Figure S4**, Supporting Information), application of recommended feature alignment settings for QTOF instruments given by MS-Dial [mean absolute deviation (MAD) of retention times ≤0.1 min, MAD of measured m/z ≤25 mDa, cf. **Table S4**, Supporting Information], calculation of the MAD of retention times, the MAD of measured m/z values, and relative standard deviation (RSD) of the integrated areas for selected features (cf. **Table S7**, Supporting Information), and limiting maximum relative standard deviation (RSD) of integrated areas for a selected feature ≤20% (cf. supporting information "Untargeted analysis – parameters and data processing"). To focus on relevant features, adducts, artifacts and in-source fragments were identified and removed with MS-CleanR,[17] and features annotated with MS-Finder.[18] Parameters applied for data processing are detailed in **Table S4** (Supporting Information, processing parameters for MS-Dial 4.7), **Table S5** (Supporting Information, MS-CleanR 1.0 with R 4.1.0 (x64)), and **Table S6** (Supporting Information, MS-Finder 3.52). From the original 16 731 collected features, 433 features were highlighted with reference hits. Statistical analysis was performed using orthogonal partial least squares discriminant analysis (OPLS-DA) and volcano plot using the R packages ropls and EnhancedVolcano.[19,20] To ensure, that replicate injections of the same sample will not be divided in the process of the cross-validation while performing the OPLS-DA, the rows (samples) of the underlying feature matrix were sorted prior to the OPLS-DA analysis, to match the specific selection pattern of the cross-validation segment selection.[41] (cf. supporting information "Untargeted analysis – parameters and data processing")

3. Results

3.1. Taste Profiles of the Selected Curry Spice Mixture, Curry Sauce, and Curry Dish for the Intervention Study

In a first set of tests, we evaluated the taste profile of the curry spice mixture used for flavoring the test meal, the sauce prepared from the mixture by boiling with coconut, freshly cut ginger, oil and salt, and the final dish as it was administered to the study participants. The sensory investigation was conducted in a consensus profiling setting after thorough panel training for the descriptors "salty," "sweet," "umami," "pungent," "velvet-astringent," "rough-astringent," and "bitter." The results for the curry spice mixture (**Figure 2**A) show that the predominant perceived taste impression was "pungent," which was rated 3/3, followed by "bitter" and "velvet-astringent," which were both rated 2/3 each. "Umami" and "rough-astringent" scored 1/3 each, and "saltiness" was not tasted (0/3). The results of the taste test of the curry sauce are provided in Figure 2B. Again, the strongest taste impression was "pungent," which was rated 2/3. "Salty" scored 1.5/3, "umami," "velvet-astringent," and "sweet" were rated 1/3, and "bitter" and "rough-astringent" were not tasted (0/3). Finally, the curry dish, consisting of curry sauce and rice (Figure 2C), was evaluated, and its taste profile was similar to that of the curry sauce with pungency as the dominant taste property: "pungent" scored 2.5/3, "salty" scored 2/3, "umami" scored 1.5/3, "velvet-astringent" and "sweet" scored 1/3, and "bitter" and "rough-astringent" were not detected (0/3).

During the preparation of the curry sauce, the ingredients of the curry spice mixture were mixed and boiled with fried fresh ginger, sunflower oil, salt, water, and coconut milk (cf. companion paper). The taste profile of the curry spice mixture was evidently heavily affected by the addition of these further ingredients, leading to the development of sweetness and saltiness, which appears to be due to the added coconut milk and sodium chloride, respectively. In contrast, bitterness and rough-astringency completely disappeared. In conclusion, pungency was the predominant oral impression of the test meal. We considered red chili, ginger, and pepper as the sources of pungent compounds. Therefore, we selected the known pungent tastants from chili capsaicin, nonivamide, dihydrocapsaicin, and 6-gingerol as the dominant pungent compound in ginger and its dehydration product 6-shoagol[21,22] and piperine from *Piper nigrum* as analytical targets of quantification in the curry spice mixture,[23] the curry sauce, and the milk samples (cf. **Figure 3**). We additionally investigated curcumin, as its bioavailability is known to be enhanced by co-administered piperine.[24]

3.2. Method Development for Targeted Quantification of Compounds from the Curry Spice Mixture

We chose HPLC-MS/MS for accurate and precise quantification of pungent compounds in the curry spice mixture, the curry sauce, and the milk samples using the two internal standards d_{10}-piperine and d_3-nonivamide. During HPLC-MS/MS method development, we individually tuned the target analytes **1**–**7** and the two internal standards for sensitive MS/MS detection and combined the parameters with a short method for chromatographic separation of analytes and the matrix using an RP18 LC column (**Table 1**). Calibration curves were prepared in neat standard solution to quantify compounds in the spice mixture and the curry sauce. The compounds **1**–**7** were calibrated in the range of 1–500 nM, providing precision of <9% relative standard deviation (RSD) and accuracy of >80%, and <110% of back-calculated standards, with the exception of compound **1** (6-

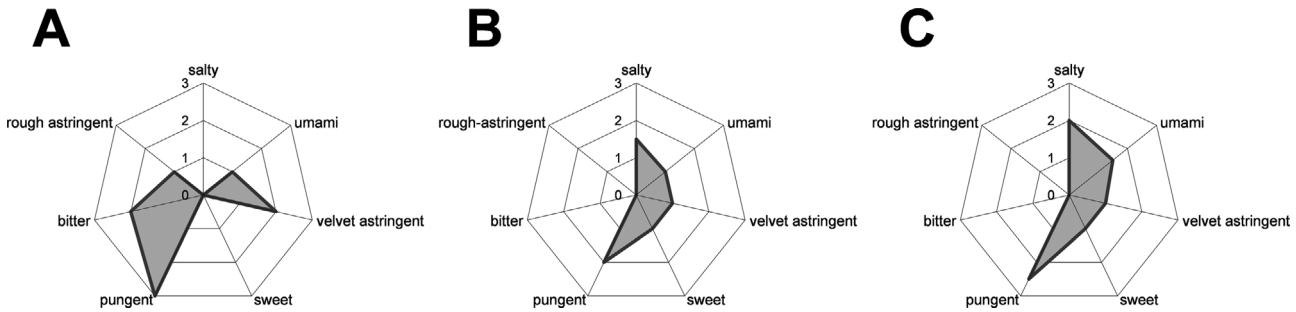

Figure 2. Consensus taste profiles ($n = 5$ participants) of (A) the mixture of spices, (B) the curry sauce, and (C) the final dish consisting of rice and the curry sauce.

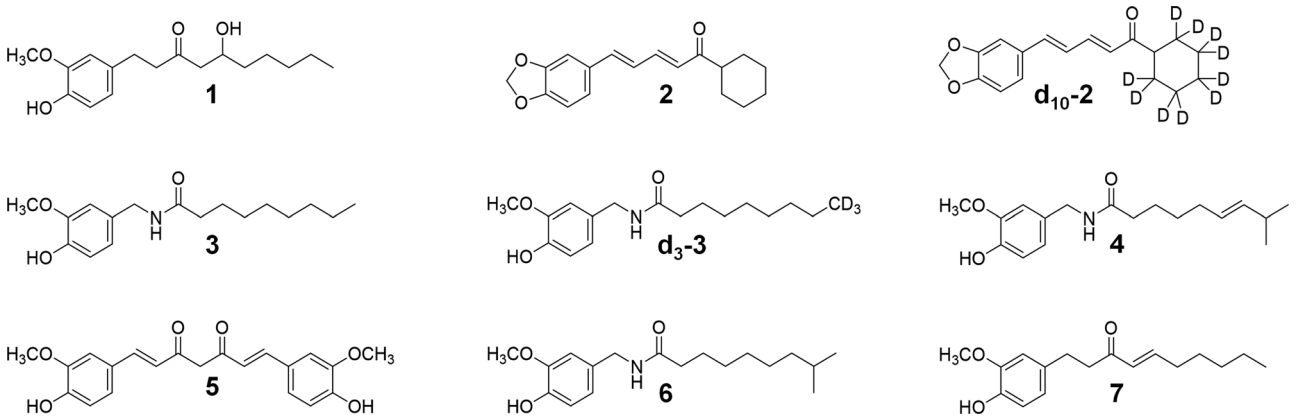

Figure 3. Structures of pungent compounds in curry and analytical standards. 1: 6-gingerol, 2: piperine, 3: nonivamide, 4: capsaicin, 5: curcumin, 6: dihydrocapsaicin, and 7: 6-shogaol.

gingerol), which had precision of <15%, accuracy of >90%, and <109% of back-calculated standards. The calibrated concentration range and the LLoQs covered the reported taste thresholds of 1–7,[25] thus ensuring concentrations sufficient as oral stimulus were quantifiable.

We prepared calibration standards using cow's milk to use a matrix similar to human milk to analyze the milk samples from the human intervention study, and we validated the method using QCs (see validation data in **Table 2**). Following a simple work-up procedure, the samples were spiked with the internal stan-

Table 1. Data on mass transitions, retention time, calibrated range, precision, accuracy, and the limit of detection and quantification of the analytes in the milk matrix.

Compound (No./IS)	Q1/Q3[a)]	Rt. [min]	Calibrated range [nM][b]	LoD/LLoQ [nM][c]	Precision [%]	Accuracy [nM]	R^2
6-Gingerol (**1**/d_3-**3**)	295/137, 177*, 94	3.60 ± 0.04	15.6–500	0.9/15.6	2.4–12.5	94–105	0.990
Piperin (**2**/d_{10}-**2**)[d]	286/201*, 115, 135	4.45, 4.59	1.9–500	0.9/1.9	2.2–8.9	93–105	0.995
Nonivamide (**3**/d_3-**3**)	294/137*, 94, 122	4.69 ± 0.01	1.9–500	0.9/1.9	2.1–7.3	91–107	0.994
Capsaicin (**4**/d_3-**3**)	306/137*, 94, 122	4.73 ± 0.01	1.9–500	0.9/1.9	2.2–11.4	90–109	0.991
Curcumin (**5**/d_3-**3**)	369/177*, 145, 285	4.75 ± 0.01	7.8–500	1.9/7.8	2.6–16.1	90–115	0.982
Dihydrocapsaicin (**6**/d_3-**3**)	308/137*, 94, 66	5.49 ± 0.01	1.9–500	0.9/1.9	1.1–12.6	92–107	0.991
6-Shogaol (**7**/d_3-**3**)[e]	277/137*, 177, 145	5.89 ± 0.01	3.9–500	0.9/3.9	2.0–15.8	96–109	0.989
d_{10}-Piperin (d_{10}-**2**)[d]	296/201*, 115, 135	4.34, 4.48					
d_3-nonivamide (d_3-**3**)	297/137*, 94, 122	4.69 ± 0.01					

[a)] MRM traces, quantifier is marked with an asterisk; [b)] calibrated range calculated using linear regression (area analyte/IS) versus concentration (analyte/IS) from triplicate injections of milk matrix standard solutions; [c)] Limit of Detection (LoD) as signal to noise ratio >3 and Lower Limit of Quantitation (LLoQ) as lowest standard concentration of the calibration curve (2 µL injection volume); [d)] piperine and d_{10}-piperine had two peaks of stereoisomers, which were not completely separated and were integrated together, we report the retention times of the separate peaks; [e)] although tuning yielded discrete fragment ions, only the quantifier ion could be observed for shogaol in standards, quality controls and samples, but not the qualifier transitions.

Table 2. Validation data for the quantification of pungent compounds in milk.

Spiking level [nM][a),b]	Compound	1	2	3	4	5	6	7
7.81	Found [nM ± SD]	-[c]	8.83 ± 0.92	8.38 ± 0.24	8.55 ± 0.90	7.55 ± 0.42	8.63 ± 1.03	8.87 ± 1.24
	RSD [%]		10.4	2.9	10.5	5.5	11.9	13.9
	Bias [%]		+13.1	+7.3	+9.5	−3.3	+10.5	+13.6
62.5	Found [nM ± SD]	63.1 ± 5.48	64.0 ± 0.90	63.1 ± 3.05	62.7 ± 4.74	62.4 ± 6.20	62.6 ± 4.15	65.4 ± 3.13
	RSD [%]	8.4	1.4	4.8	4.7	9.9	6.6	4.8
	Bias [%]	+4.2	+2.4	+0.9	+0.3	+0.2	+0.2	+4.7
500	Found [nM ± SD]	482.7 ± 38.7	485.0 ± 26.7	476.7 ± 17.2	471.0 ± 21.17	511.3 ± 36.2	470.7 ± 41.5	482.3 ± 45.8
	RSD [%]	8.01	5.5	3.6	4.5	7.1	8.8	9.5
	Bias [%]	−3.5	−3.0	−4.6	−5.8	2.3	−5.8	−3.5

[a)] The analyte solution was spiked into analyte-free cow's milk; [b)] data are means of triplicates ($n = 3$); [c)] outside calibrated range.

dards and diluted with a mixture of acetonitrile and methanol to precipitate proteins and separate the lipids. Then, we evaporated the supernatant that was obtained after centrifugation, redissolved the residue, and injected the solution into the HPLC-MS/MS. The precision of the back-calculated matrix standards was <17 % RSD, and accuracy ranged between 90% and 109 % for compounds **1**–**7**). The limits of detection, in terms of a signal to noise ratio >3 were 0.9 nM for compounds **1**–**4** and **6**–**7** and 1.9 nM for **5**. We analyzed further samples of cow's milk spiked with compounds **1**–**7** at concentrations of 7.81, 62.5, and 500 nM to serve as QCs in replicates. The precision was <14 % RSD for the lowest QC and <10% for the middle and highest QC. Bias was <±14% for the lowest QC sample and <±6 % for the other QCs (cf. Table 2). Replicate analysis ($n = 4$ per sample) of an authentic sample set (volunteer 7668, "high basal piperine group") showed precision values <10 % RSD (cf. **Table S2**, Supporting Information). Using this sample preparation and quantification method, we analyzed the milk samples collected in the human intervention study.

Notably, the purchased standard compound piperine and synthetic d_{10}-piperine had two consecutive peaks of similar height, while in the authentic milk sample, the first peak was approximately five times higher than the second one. Under standard reversed phase conditions, piperine is the first isomer to elute, followed by the UV-induced isomerization products chavicine, isopiperine, and isochavicine.[26] The two peaks were integrated as one compound-related MS/MS-signal.

3.3. Targeted Quantification of Compounds in the Curry Spice Mixture

The quantities of pungent tastants and curcumin in the curry spice mixture and final curry sauce, which was served with rice during the intervention, are presented in **Table 3**. Piperine was the most abundant tastant (3507.1 nmol g^{-1}) in the spice mixture, followed by capsaicin and capsaicinoids (nonivamide, dihydrocapsaicin), with a total of 662.8 nmol g^{-1}. In the curry sauce, which was prepared from the curry spice mixture, coconut milk, sunflower oil, and fresh ginger, the dominant compound was 6-gingerol at 464.1 nmol g^{-1}, followed by piperine (264.0 nmol g^{-1}) and capsaicinoids (25.9 nmol g^{-1}). 6-Shogaol, which is a pungent compound formed from 6-gingerol by heat-induced elimination of water,[22] was only found at 5.7 nmol g^{-1}. The data suggested that capsaicinoids were partly degraded during the preparation of the sauce, despite their relative stability.[27] The quantitative data from replicate analysis are provided in Table 3.

Table 3. Concentration of selected compounds in the curry spice mixture and the curry sauce ($n = 5$).

Compound (No.)	Curry spice mixture	Curry sauce
	c [nmol g^{-1}] in	
6-Gingerol (**1**)	9.5 ± 1.3	464.1 ± 19.7
Piperine (**2**)	3507.1 ± 326.5	264.0 ± 27.8
Nonivamide (**3**)	11.1 ± 1.1	0.7 ± 0.2
Capsaicin (**4**)	417.7 ± 36.0	15.5 ± 0.14
Curcumin (**5**)	1934.1 ± 116.7	155.7 ± 19.6
Dihydrocapsaicin (**6**)	234.0 ± 17.4	9.7 ± 0.5
6-Shogaol (**7**)	n.d.	5.7 1.0

3.4. Targeted Quantification of Compounds in Milk

Chromatograms of targeted HPLC-MS/MS analysis of pungent compounds in human milk are presented in **Figure 4** and **Figure S1** (Supporting Information). Neither 6-gingerol, 6-shogaol, nor traces of curcumin or capsaicinoids were detectable. Piperine, the primary pungent compound in black pepper, was the only analyte that was detected. The MRM traces of the internal standard d_{10}-piperine and the analyte piperine in matrix calibration standard and an authentic sample (volunteer 9192) are shown in Figure 4. The concentration ranged between 10 and 273 nM (cf. **Figure 5**, panel A). The milk samples were divided into two separate groups. The "low basal piperine group" was comprised of 11 study participants ($n = 9$ plus the additional two participants who agreed to collect additional samples). The group started at

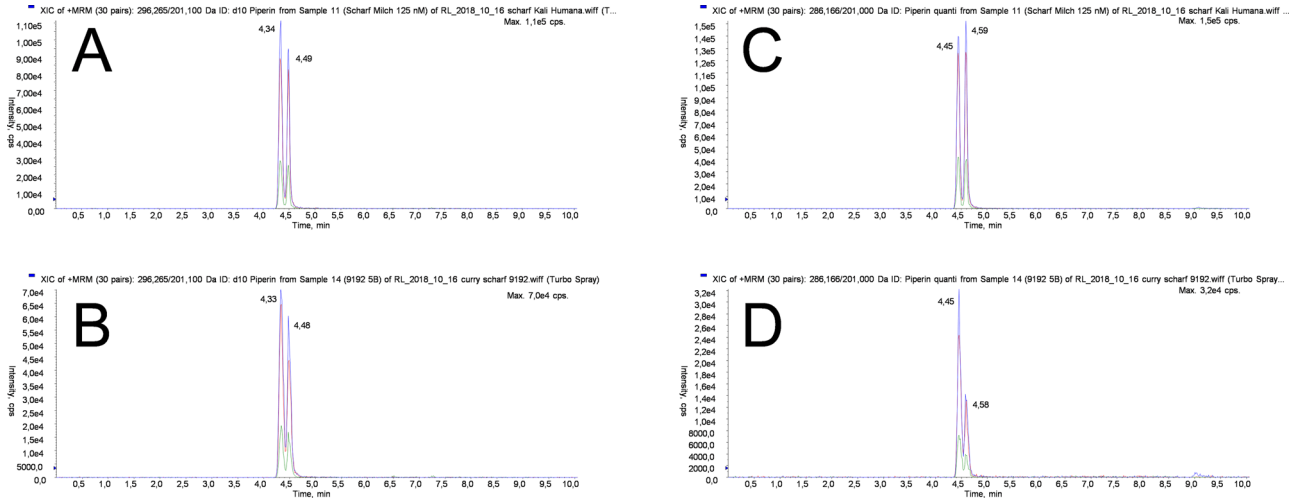

Figure 4. High performance liquid chromatography and tandem mass spectrometry analysis of piperine. MRM traces of the internal standard d_{10}-piperine in A) matrix calibration standard and B) authentic human milk (volunteer 9192), and MRM traces of the analyte piperine in C) matrix calibration standard (125 nM) and D) authentic human milk (volunteer 9192).

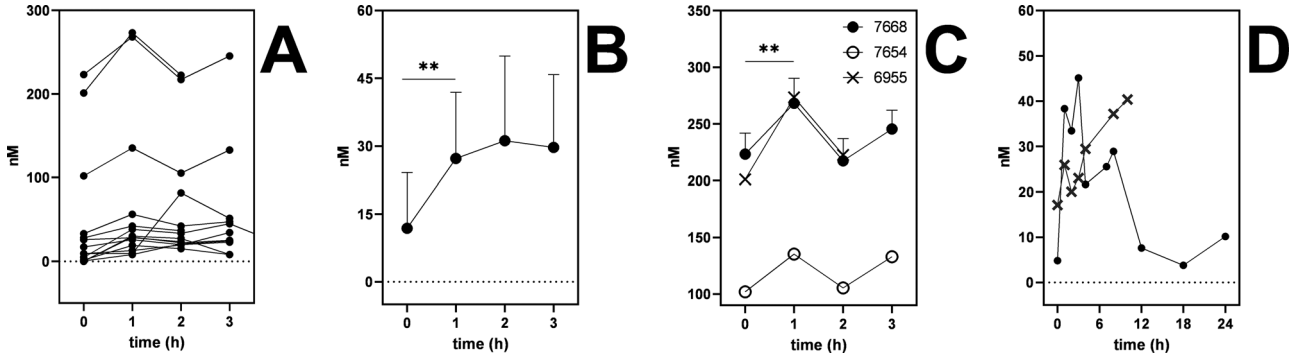

Figure 5. Concentrations of piperine in human milk before (0) and after consumption of a curry dish. A) data (means) from all individuals ($n = 11+2$); B) close-up of the "low basal piperine group" (mean ± SD of $n = 9+2$ individuals); C) close-up of the "high basal piperine group" with individual data from the participants 7668 ($n = 4$), 7654, and 6599 ($n = 1$ each); D) piperine data of collected milk samples from two mothers over an extended period (10 h, crosses and 24 h, dots).

< 15 nM (Ø 11.8 ± 12.3 nM), and ingestion of the curry dish led to an immediate significant increase of piperine in milk to 31.2 ± 18.7 nM at t = 1 h ($p < 0.01$) (see panel B). The "high basal piperine group" comprised three study participants with an initial concentration of piperine >100 nM (see panel C). Here, the piperine concentration of participant 7668 significantly increased from 223.2 ± 18.6 ($t = 0$, $n = 4$) to 268.2 ± 22.0 ($t = 1$ h, $n = 4$, $p < 0.01$, cf. Tables S2 and S3, Supporting Information). A similar trend was observed for participants 6955 and 7654 ($n =$ 1 each). Two mothers collected milk samples over an extended period after consumption of the curry dish (Figure 5, panel D, 10 h crosses, 24 h dots). In both cases, initial levels of **2** (before the meal; 5–18 nM) increased significantly ($p < 0.01$) within 1 h and then showed an immediate drop followed by a further increase. The maximum concentrations were 45.1 ± 0.7 nM (dots) reached after 4 h and 40.4 ± 1.5 nM (crosses) reached after 10 h. See **Table S3** (Supporting Information) for tabled concentrations in milk.

3.5. Untargeted Metabolomic Analysis of Human Milk Samples

In addition to the targeted analysis of compounds related to the overall oral impression of the curry dish (cf. Figure 2), untargeted MS-investigations were conducted to identify further compounds or metabolites in the milk that originated from the curry dish. The workflow for this analysis is shown in **Figure 6**. We used UPLC-separation and untargeted Triple-ToF detection (SWATH-MS, ESI^+) in the mass range of 50–1000 m/z to investigate the milk. We analyzed the milk samples taken before consumption of the test meal ("control,") and the samples collected three hours after the test meal ("treated,") along with pooled samples of each condition and two QC samples.

Repeated pre-batch injections of a QC sample prepared from aliquots of all individuals ($n = 13$, "QC13") served for instrument conditioning (cf. supporting information). QC10 prepared from all but the "high basal piperine group" (6955, 7654, and 7668) ($n = 10$, "QC10") was injected every as 10th sample in the batch

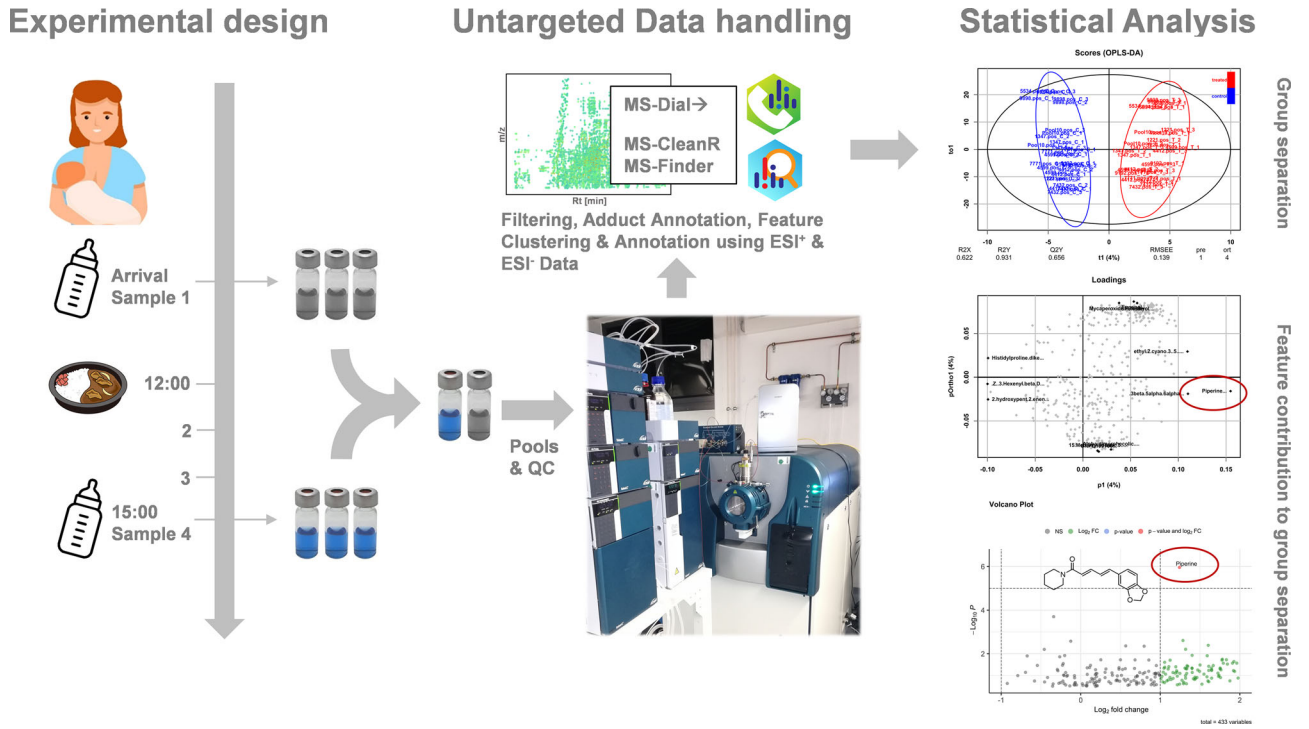

Figure 6. Workflow for compound identification: experimental design of human intervention study (left), data curation and handling of untargeted data (middle), and subsequent statistical analysis and visualization for identification of contributors to group differences (right: OPLS-DA for separation of "control" and "treated," loadings plot and volcano plot for feature-contribution and marker identification). Figure 6 will be substituted by a higher resolution figure during submission of manuscript.

and used for drift normalization and alignment in MS-Dial. Prior to the statistical analysis of the dataset, the amount of features was reduced using MS-CleanR,[17] which uses the MS-Dial output to perform blank signal subtraction and filtering steps based on the relative standard deviation (RSD) across sample classes, the relative mass defect (RMD) and clustering of features, to preserve only unique features. Adducts, artifacts, and in-source fragments are thus removed from the dataset. This approach also allows an adduct annotation correction by importing feature alignment matrices in both ionization modes, which improves the further feature annotation. The originally collected features were reduced from 16 731 to 433 (cf. Figure 6, cf. Supporting Information).

For the further statistical analysis, the reduced feature alignment matrix was used, and all samples (workup triplicates) and pooled samples (injection triplicates) allocated to the "control" group ("before curry consumption") and to the "treated" group ("after curry consumption"). Performing a first OPLS-DA indicated subject 3321 as an outlier why this subject was removed from the calculation of the second OPLS-DA (cf. Supporting Information). By inspecting the loadings plot (Figure 6, **Figure 8**, Supporting Information), only piperine was detected far on the right side representing the samples "after curry consumption." These findings were verified by calculating *p*-values and $\log_2$ fold changes based on the reduced feature matrix. The volcano plot (Figure 6, **Figure S9**, Supporting Information) highlighted piperine as the only compound in human milk as abundant above the default threshold settings for the p-value ($10e^{-6}$) and log2 fold change (2) (cf. Figure 6).

3.6. Human Sensory Threshold in Milk

Both untargeted and targeted MS-investigations identified piperine as a dietary flavor compound in human milk after ingestion of a standardized curry dish. However, the impact of human milk on the taste properties of this pungent compound is unknown. Therefore, we investigated whether the concentrations quantified in the milk are detectable by the human tongue. As a matrix, we chose a commercially available follow-on formula warmed to 37°C before sensory evaluation to keep as close to the composition of authentic human milk as possible. A concentration of 8.75 µM piperine was not distinguishable from unspiked follow-on formula in a triangle setup. However, the piperine-containing sample was detected at 17.5 µM (α-level = 0.2, $n = 7$, cf.[13]), with two participants detecting slight pungency, while the other three detected a vague sensory difference to the unspiked samples. This estimated threshold was within the range of taste data without matrix reported earlier.[25] Therefore, we estimate that a specific identification of piperine in milk by the oral impression of "pungency" requires a much higher concentration than 17.5 µM. We emphasize that the highest piperine concentration found in milk (273.8 nM, volunteer 6955, cf. **Table S3**, Supporting Information) was ≈70 times lower (Figure 5, panel C). In conclusion, it

appears improbable that nurslings experience the pungent taste of piperine during breastfeeding.

4. Discussion

We hypothesized that dietary flavor compounds could enter human milk and be transferred to a child during nursing. A possible consequence of this could be the programming of sensory reactions, including preferences, such as for sweet or savory taste impressions, or alleviated refusal of a bitter or pungent oral impression (e.g.,[28]). The aim of the experiments was to investigate whether taste compounds present in a test meal were transferred to the milk of nursing mothers and could come in contact with a nursing child.

We found a high abundance of 6-gingerol in the sauce, which originated from freshly cut up ginger in the sauce (cf. Table 3). However, neither untargeted nor targeted UPLC-MS analyses identified 6-gingerol in the human milk. Despite its quick bio-appearance after oral administration, no free 6-gingerol is detectable in plasma or urine after ginger consumption,[29] because it is quickly connected to glucuronic acid in phase 2-metabolism.[29,30] Heat leads to the elimination of water from 6-gingerol and the formation of shogaol (**7**).[22] Table 3 shows that despite the frying process of ginger and subsequent boiling of the sauce, no substantial amounts of 6-shogaol were formed. Additionally, neither targeted nor untargeted UPLC-MS experiments led to the detection of free 6-shogaol in milk, either due to metabolic breakdown or hindered diffusion. Capsaicinoids (compounds **3**, **4**, and **6**) were abundant in the curry spice mixture, with a total of ≈660 nmol g^{-1}. However, during the preparation of the curry sauce, the concentration showed a substantial drop to ≈27 nmol g^{-1} (cf. Table 3). While capsaicinoids are stable up to 100°C, elevated temperatures >190°C lead to rapid losses due to water vapor volatility and decomposition, reaching almost quantitative degradation (>90%) during roasting or frying processes.[27] The preparation of the curry sauce involved a standardized procedure, which exposed the ingredients to 5 min of 100°C. Evidently, this was enough to reduce the content of nonivamide, capsaicin, and dihydrocapsaicin to only small residues. In rodents, capsaicin is bioavailable through passive diffusion, with peak plasma concentrations after ≈1 h.[31] However, the small amounts of capsaicinoids putatively present in the vascular systems of the study participants did not diffuse into the milk. In future studies, the analysis of synchronically collected blood and milk samples could contribute to the clarification of this observation.

The physicochemical and health-related properties of piperine have recently been reviewed.[32] A unique characteristic of piperine is that it is bioavailable from the diet despite its low solubility in water, and it enhances the bioavailability of other compounds, such as drugs, toxins, and food compounds, making it a feasible vehicle for therapeutic uses. Curcumin was present in the final curry sauce at ≈155 nmol g^{-1}, and it has been reported to show increased oral bioavailability in rodents when piperine was co-administered.[31] Therefore, we suspected curcumin in the milk samples but did not detect any.

Although the preparation process reduced the amounts of piperine from ≈3500 nmol g^{-1} in the curry spice mixture to 264 nmol g^{-1} in the sauce, ingestion of the dish led to ≈50 nM piperine in the human milk (Figure 5). The observation that milk from three of the study participants showed a substantial piperine concentration before ingestion of the curry dish (cf. **3.4**) was surprising because piperine is not a human metabolite but of strictly herbal origin. However, we assumed that this finding was because pepper is a commonly used spice. Dietary records corroborated this suspicion, as some of the foods consumed within the washout phase were Bavarian meat dishes, pizza, stews, and soups, which are usually flavored with salt and pepper.

In the time-resolved investigations over an extended time period (Figure 5, panel D), the milk samples collected ≈1 h after consumption of the test meal already showed a sharp increase in piperine. Piperine reached its maximum (45.1 nM) after 3 h in the milk of volunteer 0007 (dots in Figure 5, panel D), and in the milk of volunteer 9192 (crosses in Figure 5, panel D) the maximum piperine concentration (40.4 nM) was 10 h after consumption of the curry dish. The data underline the inter-individual time span in response to the piperine dose, and it is evident that both uptake and transfer into the milk are not immediate and probably affected by other variables. We conducted the workflow outlined in Figure 6 with the aim to identify possible other compounds originating from the curry dish using untargeted MS-analysis. The results, however, corroborated the initial results of targeted MS-quantitation, which found piperine as the only significant contributor to group differences (cf. supporting information).

Piperine was first qualitatively identified in human milk by Khachik et al.[10] who analyzed carotenoids in human milk and serum. Our untargeted and targeted quantitative metabolomics results corroborate their findings and add quantitative data to the literature. Piperine activates the vanilloid receptor-1 (TRPV1). In adults Dawid et al.[23] reported 3 nmol cm^{-2} (applied on filter paper as vehicle) as the recognition threshold. We estimated the threshold in follow-on formula to be ≥17.5 µM. Compared to this concentration, the concentration we detected in milk (up to 273 nM, cf. **Table S2** and **S3**, Supporting Information) was so low that we consider it unlikely to be consciously perceived by the nursling, except if infants are more sensitive. However, piperine will nevertheless interact with the TRPV-receptors of the child during nursing. McNamara et al.[33] reported that piperine desensitizes the TRPV1. Therefore, it appears plausible that frequent exposure to piperine through human milk containing sub-taste-threshold concentrations could contribute to an increased tolerance at a later age.

Considering the observation that none of the selected analytes of the test meal except piperine was identified in milk, we hypothesize a barrier between the mother's circulation and the mammary glands that, in the present scenario, only piperine could traverse. This seems comparable to the blood brain barrier (BBB), which regulates and restricts access of compounds to the brain for protection and to enable proper functionality.[34] Such a protection mechanism makes sense from an evolutional standpoint because the nursling needs the maternal nutrient supply to grow and develop, and it needs protection from hazardous compounds. Interestingly, Khachik et al.[10] identified caffeine as a second compound, after piperine, of dietary origin in human milk. Caffeine is a compound known to cross the BBB.[35] Both caffeine and piperine are lipophilic, low molecular weight compounds, which are two characteristics that favor BBB crossing.[36] BBB permeability predictions for piperine and analogs from in vitro models and experimental permeability data from animal

studies have shown that piperine diffuses into the brain after oral administration.[37,38] Given the evidence that both caffeine and piperine cross the BBB and are detectable in human milk after oral ingestion suggests that compounds that cross the BBB could be found in human milk and vice versa. Interestingly, the phenomenon of increased bioavailability of curcumin when piperine was co-administered did not lead to an abundance of curcumin in human milk.

5. Concluding Remarks

In conclusion, piperine was the only tastant that was detected in human milk after ingestion of the curry dish. However, its concentration was assumed to be too low for conscious detection by the nursling, and it could contribute to a sensory desensitization toward pungency in later life. In this context it must be noted that the collected samples were either foremilk or a mixture of fore- and hindmilk (cf. Table S1 Supporting Information), respectively. Given its lipophilicity, it appears possible, that piperine might accumulate in hindmilk, which usually contains more lipids than foremilk.[40]

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

K.N. did quantitative analysis, untargeted data acquisition, data analysis, sensory experiments, and wrote the manuscript. M.D. realized the human intervention study, provided samples, and wrote the manuscript. J.B. did untargeted data processing and wrote the manuscript. S.D. did untargeted data processing and wrote the manuscript. TD did sensory experiments and contributed to the manuscript. A.B.e. contributed to synthesis, targeted and untargeted analysis, and the manuscript. V.S. contributed to synthesis, targeted and untargeted analysis, and the manuscript. C.D. synthesized compounds and contributed to the manuscript. HL conceptualized the project, supervised the human intervention study, and wrote the manuscript. A.B.u. conceptualized und supervised the project and contributed to data interpretation and the manuscript. R.L. synthesized compounds, developed targeted MS-methods, did quantitative analysis, data interpretation, and wrote the manuscript. TH conceptualized und supervised the project and contributed to data interpretation and the manuscript.

Data Availability Statement

Targeted data are included in the supporting information. Untargeted data are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Keywords

human milk, metabonomics, piperine, sensory programming

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