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Metabolites of 2- and 3-Monochloropropanediol (2- and 3-MCPD) in Humans: Urinary Excretion of 2-Chlorohydracrylic Acid and 3-Chlorolactic Acid after Controlled Exposure to a Single High Dose of Fatty Acid Esters of 2- and 3-MCPD

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Scope: Fatty acid esters of 2-monochloropropane-1,3-diol (2-MCPD) and 3-monochloropropane-1,2-diol (3-MCPD) are formed during the deodorization of vegetable oils. After lipase-catalyzed hydrolysis in the intestine, 2- and 3-MCPD are absorbed, but their ensuing human metabolism is unknown.
Methods and results: The compounds 2-chlorohydracrylic acid (2-ClHA) and 3-chlorolactic acid (3-ClLA) resulting from oxidative metabolism of 2-MCPD and 3-MCPD, respectively, are identified and quantified in human urine samples. An exposure study with 12 adults is conducted to determine the urinary excretion of 2-ClHA and 3-ClLA. The participants eat 12 g of hazelnut oil containing 24.2 mg kg^{-1} 2-MCPD and 54.5 mg kg^{-1} 3-MCPD in the form of fatty acid esters. Average daily amounts of "background" excretion before the exposure are 69 nmol 2-ClHA and 3.0 nmol 3-ClLA. The additional mean excretion due to the uptake of the hazelnut oil amounts to 893 nmol 2-ClHA (34.0% of the 2-MCPD dose) and 16.4 nmol 3-ClLA (0.28% of the 3-MPCD dose).
Conclusions: The products of oxidative metabolism of 2- and 3-MCPD, 2-ClHA, and 3-ClLA, are described for the first time in humans. Due to the lack of specificity, the metabolites may not be used as exposure biomarkers to low doses of bound 2- and 3-MCPD, respectively.

1. Introduction

The compounds 2-monochloropropane-1,3-diol (2-MCPD) and 3-monochloropropane-1,2-diol (3-MCPD) were detected first in thermal protein hydrolysates, for example, soy sauce.[1] Their fatty acid esters are common food-borne contaminants usually generated by thermal processing (deodorization) of vegetable oils and fats,[2] which are consumed directly or used for the production of other foods, for example, infant formula as well as many other food classes.[3] The major part of the fatty acid esters of 2- and 3-MCPD are hydrolyzed efficiently in the intestinal tract to release 2- and 3-MCPD which are more or less completely absorbed.[4–6]

The target organs of 3-MCPD toxicity in animals are kidneys, testes, and mammary glands. In two chronic studies, progressive nephropathy accompanied by hypertrophy of the kidney (male and female rats), glandular hyperplasia in the mammary gland (male rats),[7] and atrophy and arteritis of the testes[8] were observed. The data on 2-MCPD toxicity in animals is limited. In a 28-day study in rats, relatively high doses (16 or 30 mg kg^{-1} body weight [bw] per day) led to cardiac failure caused by severe myopathy and nephrotoxicity.[9] There is no data on the toxicity of 2- and 3-MCPD in humans.[3]

The carcinogenicity of 3-MCPD was studied in different 2-year bioassays in mice and rats ingesting 3-MCPD with the drinking water. In F344 rats, the incidences of adenomas in renal tubules (both sexes), in the Leydig cells, and in the mammary gland (male rats) were increased.[7] In a later 2-year bioassay with Sprague–Dawley rats, 3-MCPD also induced renal tubule carcinomas (in males) and Leydig cell tumors.[8] There is no data on 2-MCPD induced carcinogenic effects in animals and on 2- or 3-MCPD carcinogenic effects in humans. Based on the data from animal experiments the International Agency for Research on Cancer (IARC) has classified 3-MCPD as possibly carcinogenic to humans (group 2B).[10] The causes of the observed carcinogenicity in animals, however, are unclear. Because there is no indication for a direct genotoxic effect in vivo,[11] the CONTAM Panel (Panel on Contaminants in the Food Chain) of the European Food

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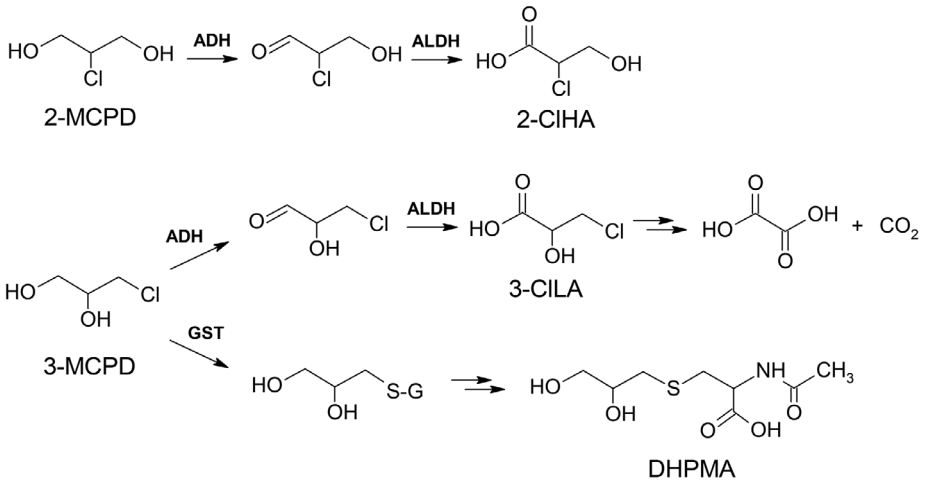

Figure 1. Metabolic pathways of 2-MCPD and 3-MCPD. The current work showed that 2-MCPD is oxidized in humans to yield 2-chlorohydracrylic acid (2-ClHA). 3-MCPD is oxidized in the same manner to 3-chlorolactaldehyde and 3-chlorolactic acid (3-ClLA), which was shown to be converted into oxalic acid and carbon dioxide in rats.[18] The glutathione conjugate leads to the formation of 2,3-dihydroxypropylmercapturic acid (DHPMA). ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; GST, glutathione S-transferase.

Safety Authority (EFSA) derived a tolerable daily intake (TDI) of 2.0 μg kg^{-1} bw, based on the most sensitive endpoint, tubular hyperplasia, observed in long-term studies in rats.[12] In contrast to 3-MCPD, the toxicological data for 2-MCPD and its fatty acid esters are too limited to derive a TDI.

The assessment of the human health risks resulting from 2- and 3-MCPD exposure is hindered because of two reasons. First, the content as well as the individual consumption of food items containing 2- and 3-MCPD vary greatly. It would be advantageous, if the uptake of 2- and 3-MPCD could be determined by quantification of urinary metabolites serving as biomarkers of exposure. Second, there is no data on the metabolism of both compounds in humans and the modes of action are basically unknown. In rats treated with high doses of 3-MCPD or its fatty acid esters, major urinary metabolites were identified as free 3-MCPD,[13] 3-chlorolactic acid (3-ClLA),[14] and *N*-acetyl-*S*-(2,3-dihydroxypropyl)cysteine (2,3-dihydroxypropylmercapturic acid; DHPMA),[5] which was hypothesized to be formed after glutathione conjugation (**Figure 1**). The primary goals of the current study were to clarify, whether 3-MCPD can be converted into 3-ClLA in humans, whether the oxidative pathway may also convert 2-MCPD to the putative metabolite 2-chlorohydracrylic acid (2-ClHA), and whether it is possible to use these substances as potential biomarkers for human exposure assessment. To this end, we developed an ion-pair liquid chromatographic (IPC) method for the separation of the isomeres 2-ClHA and 3-ClLA and an isotope-dilution technique using tandem-mass spectrometry (MS/MS) for their quantification. The method was subsequently applied to the analysis of human urine samples from a controlled exposure study, in which 12 participants ate 12 g of a 2-/3-MCPD ester-rich hazelnut oil.[15]

2. Results

2.1. Detection of 2-ClHA and 3-ClLA

Considering the characteristic ratio of stable chlorine isotopes, ^{35}Cl (34.96885 atomic mass u^{-1}, 75.77%) and ^{37}Cl (36.96590 atomic mass u^{-1}, 24.23%),[16] a mass spectrometric product ion scan allows detecting specifically compounds losing chloride ions by collision-induced dissociation (CID). In search of 3-ClLA, an acknowledged metabolite of 3-MCPD in rats, urine samples from the human study participants collected after controlled exposure to thermally processed hazelnut oil were screened by CID in the negative ESI mode for compounds characterized by the loss of the chlorine-specific ion fragments of $m/z = 35$ and $m/z = 37$. Two peaks of parent ions were observed with $m/z = 123$ and $m/z = 125$ at relatively short retention times (**Figure 2**A). The area ratio of the transitions $m/z = 123 \rightarrow 35$ and $m/z = 125 \rightarrow 37$ of both peaks corresponded to the expected isotope-ratio of chlorine (≈3). The commercial compound 3-ClLA co-eluted with the peak 2 of the chromatograms. Another signal with relatively high intensity (peak 1 in Figure 2A) indicated the presence of another chlorine-containing compound, probably with the same atomic composition as 3-ClLA ($C_3O_3H_5Cl$). We suggested that it was 2-ClHA, a regioisomer of 3-ClLA, resulting from the oxidation of 2-MCPD. The identity was confirmed after extraction by determining the exact masses of the parent molecule and of the fragments (Figure 2B) and, in addition, by LC-MS/MS analysis of the commercially available standard substance.

2.2. Isotope-Dilution LC-MS/MS for the Quantification of 2-ClHA and 3-ClLA

Various methods were tested in order to enrich the analytes and to reduce the urinary matrix, that is, solid-phase extraction using Oasis HLB or Oasis MAX columns and liquid–liquid extraction methods using different solvents (data not shown). The best recovery of 2-ClHA and 3-ClLA was achieved with the liquid–liquid extraction from the urine samples at pH = 2 with ethyl acetate. On the basis of the fragments with specific mass-to-charge ratios of $m/z = 35$ and $m/z = 37$, we devised an isotope-dilution technique for the purpose of 2-ClHA and 3-ClLA quantification in the urinary extracts using MRM and defined amounts of [$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA as internal

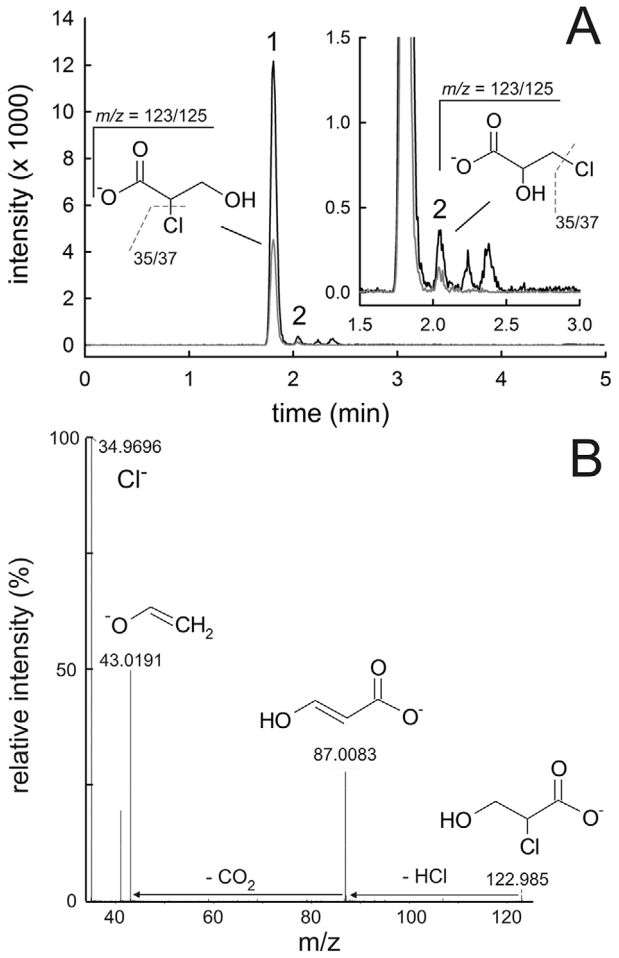

Figure 2. An extract of human urine collected 2–4 h after the consumption of 12 g hazelnut oil containing 24.2 mg kg^{-1} 2-MCPD and 54.5 mg kg^{-1} 3-MCPD was screened with a product ion scan in the negative ESI mode (System I) for compounds dissociating chloride ions ($m/z = 35$ and $m/z = 37$). A) The chromatograms were from the transitions $m/z = 123 \rightarrow 35$ (black line) and $m/z = 125 \rightarrow 37$ (grey line). Using commercially available compounds as reference, the peaks were assigned to 2-ClHA (peak 1) and 3-ClLA (peak 2; amplification in the inset). B) The exact mass and the MS^2 fragment ion spectrum of peak 1 in panel A in the negative mode confirmed the assignment.

standards. Both compounds were monitored simultaneously by equivalent fragmentation reactions after ionization in the negative mode: the abstraction of the chloride ion from the analytes resulting in two signals from the transitions $m/z = 123 \rightarrow 35$ (quantifier signal) and $m/z = 125 \rightarrow 37$. However, the inadequate chromatographic separation of both analytes by conventional reversed-phase chromatography (System I) caused problems, especially in extracts of urine samples collected some hours after the exposure to the hazelnut oil. In these samples, the detection of 3-ClLA was hindered because the baseline separation decreased with the increasing intensity of the 2-ClHA peak. As a result, the signal-to-noise ratio of the relatively small signal of 3-ClLA was lowered. The problem was solved using an IPC-method for the chromatographic separation of 2-ClHA and 3-ClLA with tributylamine as ion pairing reagent.[17] Figure S2, Supporting Information depicts the improvement of the analysis with the IPC method (System II) relative to the results achieved by conventional reversed-phase chromatography (System I).

Table 1. Validation parameters for the analyses of [$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA in human urine samples.[a)]

			[$^{13}C_3$]2-ClHA	[$^{13}C_3$]3-ClLA
Without matrix				
Linear detection range[b)]		fmol per col.	10.9–21 800	3.1–31 400
LOD[b)]		fmol per col.	10.9	3.1
LOQ[b)]		fmol per col.	21.8	15.7
With matrix				
Linear detection range[b)]		fmol per col.	21.8–21 800	6.3–31 400
LOD[b]		fmol per col.	21.8	6.3
LOQ[b]		fmol per col.	109	31.4
Mean relative recovery[c)]		%	45.7 ± 1.8	53.0 ± 2.7
Intraday precision (CV)[d)]	low	%	2.6	12.3
	medium	%	2.9	4.7
	high	%	2.5	3.9
Interday precision (CV)[d)]	low	%	14.6	12.8
	medium	%	5.1	4.2
	high	%	4.6	3.3

[a)] The validation parameters were determined using isotope-labeled compounds due to the high background of urinary 2-ClHA and 3-ClLA; [b)] The linearity of detection and the LOD and LOQ values were determined using 14 solutions of [$^{13}C_3$]2-ClHA (0.14 to 2720 nM) and [$^{13}C_3$]3-ClLA (0.196 to 3920 nM), which were either prepared in water or in the presence of biomatrix extracted from blank samples of pooled urine (Figure S4, Supporting Information). The LOD and LOQ were defined by a S/N > 3 and a S/N > 10, respectively; [c)] The mean recovery of the extraction was determined with three different concentrations of [$^{13}C_3$]2-ClHA (10.9, 54.4, or 272 nM) and [$^{13}C_3$]3-ClLA (15.7, 78.4, or 392 nM); [d)] The precision was determined using spiked urine samples with nominal concentrations 8.03 nM (low), 80.3 nM (medium), and 803 nM (high) 2-ClHA and 3-ClLA.

2.3. Validation

The isotope purity of the standards was determined by separate injections of 18.6 pmol [$^{13}C_3$]2-ClHA or 31.4 pmol [$^{13}C_3$]3-ClLA on column (Figure S3, Supporting Information). The minimal peak in the analyte trace ($m/z = 123 \rightarrow 35$) at the retention time of 3-ClLA did not pose a problem for the quantification of 3-ClLA in urine samples when amounts of 3.14 pmol of [$^{13}C_3$]3-ClLA/injection were applied as internal standard. There was no 2-ClHA detectable when 18.6 pmol of [$^{13}C_3$]2-ClHA on column were analyzed. Even after the injections of 18.6 pmol of [$^{13}C_3$]2-ClHA or 31.4 pmol of [$^{13}C_3$]3-ClLA on column, a carryover of the chromatographic method was not observed.

The linearity of detection and the values for limit of detection (LOD) and limit of quantification (LOQ) were determined using 14 solutions of [$^{13}C_3$]2-ClHA (0.14 to 2720 nM) and [$^{13}C_3$]3-ClLA (0.196 to 3920 nM), which were either prepared in water or in the presence of biomatrix extracted from blank samples of pooled urine (Figure S4, Supporting Information). LOD and LOQ values of [$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA (**Table 1**) are only approximates for those of the unlabeled analytes. Because of the varying "background" in the urine samples, we used the S/N of the individual signals for the judgment of the detection (S/N > 3)

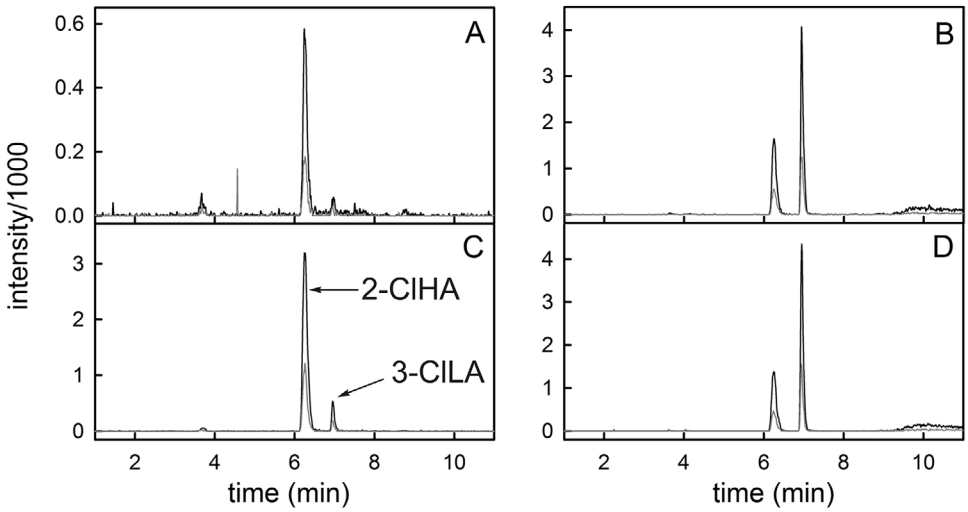

Figure 3. Analysis of 2-ClHA and 3-ClLA by IPC-MS/MS MRM (System II) in the urine samples of an exemplary participant A,B) before and C,D) 4 h after consumption of the hazelnut oil. The panels on the left (A and C) show the MRM traces of the analytes 2-ClHA and 3-ClLA with both transitions $m/z = 123 \rightarrow 35$ (black lines, quantifier signal) and $m/z = 125 \rightarrow 37$ (grey lines) recorded in the negative ESI mode. The panels on the right (B and D) show the transition resulting from the addition of 27.2 pmol [$^{13}C_3$]2-ClHA and 39.2 pmol [$^{13}C_3$]3-ClLA to the urine samples before the extraction ($m/z = 126 \rightarrow 35$ [black lines] and $m/z = 128 \rightarrow 37$ [grey lines]).

and the quantification (S/N > 10). The results of the intra- and interday precision determined with three different concentrations of 2-ClHA and 3-ClLA are also summarized in Table 1.

2.4. Human Controlled Exposure Study

Eight urine samples of each of the participants were collected in the 48 h before the controlled exposure to the hazelnut oil and 14 urine samples in the 48 h afterward. Representative chromatography data for the quantification of 2-ClHA and 3-ClLA in two urine samples of one participant, collected before and after the controlled exposure, are depicted in **Figure 3**. The concentrations of 2-ClHA and 3-ClLA were determined in all urine samples and the mean excretion rate was calculated for each time interval. After the controlled exposure to the hazelnut oil, an instantaneous increase of the urinary excretion rates of 2-ClHA and 3-ClLA was observed (**Figure 4**). The highest mean excretion rates of 2-ClHA and 3-ClLA were reached in the time intervals from 5 to 6 h (range between 4 to 5 h and 8 to 12 h) and from 4 to 5 h (range between 1 to 2 h and 7 to 8 h), respectively, after ingestion of the hazelnut oil. The mean elimination half-lives of 2-ClHA ($t_{1/2}$ = 8.2 h) and 3-ClLA ($t_{1/2}$ = 3.8 h) were determined by linear fitting of the time points after the maximum excretion rate plotted on a half-logarithmic ordinate scale (Figure S5, Supporting Information).

Tables 2 and **3** summarize the individual daily excretion of 2-ClHA and 3-ClLA, respectively, for all of the participants in the course of the study. On the "background" days 1 and 2, average urinary excretion was 82 and 57 nmol in case of 2-ClHA, respectively. After subtraction of the average individual daily "background" excretion, the additional amount excreted after the controlled exposure (mean ± SD) was 716 ± 170 nmol 2-ClHA on day 3 and 176 ± 78 nmol 2-ClHA on day 4 of the study, corresponding to 27.2 ± 6.5% and 6.7 ± 3.0% of the 2-MCPD dose, respectively (in total: 893 ± 159 nmol 2-ClHA corresponding to

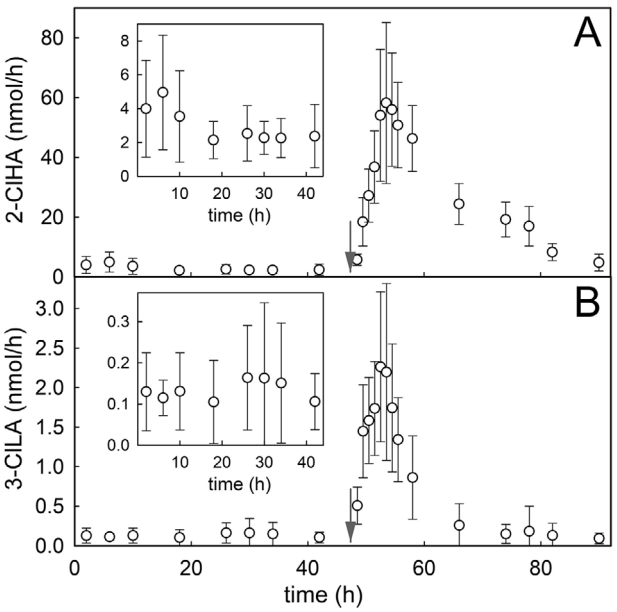

Figure 4. Mean hourly excretion of A) 2-ClHA and B) 3-ClLA of the 12 study participants (± SD) two days before (days 1 and 2) and two days after the exposure (days 3 and 4) to 12 g hazelnut oil containing 2-MCPD (290 µg, 2627 nmol) and 3-MCPD (654 µg, 5917 nmol). The arrows mark the time of controlled exposure on Wednesday morning and the insets are amplifications of the "background" excretion data.

34.0 ± 6.0% of the 2-MCPD dose). The half-life time of 2-ClHA ($t_{1/2}$ = 8.2 h) indicated that the excretion monitored until the end of day 4 yielded only about 98% of the overall elimination of 2-ClHA. In case of 3-ClLA, the mean amount excreted on day 4 of the study was about the same (≈3 nmol day^{-1}) than those observed on the two "background" days before the controlled exposure (Table 3). Obviously, the elimination of the 3-ClLA formed

Table 2. Urinary excretion of 2-ClHA of each participant on the four days of the study and estimated additional excretion following uptake of hazelnut oil (2627 nmol 2-MCPD) on the morning of day 3.

Participant	Excretion of 2-ClHA					
	Day 1 [nmol]	Day 2 [nmol]	Day 3 [nmol]	Day 4 [nmol]	Days 3 + 4 (1 + 2)	
					[nmol]	[% of dose]
A	32	52	1120	171	1208	46.0
B	65	22	875	183	970	36.9
C	77	58	925	260	1050	40.0
D	109	37	760	220	833	31.7
E	42	37	903	163	988	37.6
F	112	30	570	185	613	23.3
G	47	30	580	337	840	32.0
H	145	128	846	193	765	29.1
I	113	104	719	327	830	31.6
J	47	48	714	371	990	37.7
K	66	66	800	216	885	33.7
L	124	70	613	320	739	28.1
Mean ± SD	82 ± 38	57 ± 32	785 ± 161	246 ± 74	893 ± 159	34.0 ± 6.0

Table 3. Urinary excretion of 3-ClLA of each participant on the four days of the study and estimated additional excretion following uptake of hazelnut oil (5919 nmol 3-MCPD) on the morning of day 3.

Participant	Excretion of 3-ClLA					
	Day 1 [nmol]	Day 2 [nmol]	Day 3 [nmol]	Day 4 [nmol]	Days 3 + 4 (1 + 2)	
					[nmol]	[% of dose]
A	1.6	2.4	22.4	0.9	19.3	0.33
B	3.7	1.7	28.3	1.4	24.3	0.41
C	3.9	3.2	37.7	6.0	36.7	0.62
D	2.4	2.7	18.1	3.6	16.6	0.28
E	2.4	2.0	16.2	0.7	12.4	0.21
F	2.7	1.7	8.4	1.3	5.2	0.09
G	1.2	6.3	19.7	9.9	22.0	0.37
H	2.8	5.0	13.2	1.9	7.4	0.12
I	1.8	3.3	18.4	1.4	14.7	0.25
J	5.0	2.4	16.8	2.2	11.7	0.20
K	5.5	6.0	15.5	4.6	8.6	0.14
L	1.0	1.5	17.9	2.4	17.8	0.30
Mean ± SD	2.8 ± 1.4	3.2 ± 1.7	19.4 ± 7.5	3.0 ± 2.7	16.4 ± 8.7	0.28 ± 0.15

from the 3-MCPD portion in the hazelnut oil consumed on the morning of day 3 was completed within 24 h. The mean amount of 3-ClLA excreted and corrected with the individual "background" levels was 16.4 ± 8.7 nmol 3-ClLA, corresponding to 0.28 ± 0.15% of the 3-MCPD dose taken up with the hazelnut oil.

The inter-individual variation of the excretion rates was much lower in case of 2-ClHA (CV = 17.9%) compared to 3-ClLA (CV = 52.9%). The individual excretion rates for 2-ClHA and 3-ClLA correlated with R^2 = 27.0%. No significant correlations were observed between the excretion rates of 2-ClHA or 3-ClLA and age, body weight, body mass index, or gender.

3. Discussion

More than forty years ago, the metabolism of 3-MCPD was studied in rats using [^{14}C]3-MCPD or [^{36}Cl]3-MCPD, which led to the detection of radioactively labeled 3-MCPD (≈8.5% of the dose) and 3-ClLA (≈23%) in the urine, and carbon dioxide (≈30%) in exhaled air.[13,14] In addition, the conjugate *S*-(2,3-dihydroxypropyl)cysteine and the mercapturic acid DHPMA were detected. In a more recent 90-days study in rats, three urinary treatment-related compounds were detected after daily application of three different doses of 3-MCPD. The highest dose of 29.5 mg kg^{-1} bw led to a mean excretion of 2.6% 3-MCPD, 12.5% DHPMA and 0.9% 3-ClLA in male rats and 5.8% 3-MCPD, 7.3% DHPMA and 1.7% 3-ClLA in female rats.[5] The current state of knowledge about 3-MCPD metabolism is summarized in Figure 1.

Regarding humans, the urinary excretion of 2- and 3-MCPD was investigated after controlled exposure to hazelnut oil and palm fat (same study, but not considered in the current work).[15] After consumption of the hazelnut oil, mean excretion rates of 2- and 3-MCPD were 14.3% and 3.7% of the study doses. The urinary excretion of DHPMA was found at a relatively constant and high rate of 1.65 µmol per day on average, without a detectable increase on day 3. This indicates an endogenous origin of DHPMA with a urinary excretion not reflecting the external exposure to fatty esters of glycidol or 3-MCPD in the "background" range.[15]

Another aim of the study was to clarify whether 3-ClLA is also a human metabolite of 3-MCPD. However, the mean amount of 3-ClLA excreted by the 12 adult participants was only 0.28% of the 3-MCPD dose ingested with the hazelnut oil. A structural isomer of 3-ClLA, the chlorine-containing compound 2-ClHA with the same fragmentations (m/z = 123 → 35 and m/z = 125 → 37) and a somewhat shorter retention time, was identified as a putative product of oxidative 2-MCPD metabolism. The high average total excretion of 2-ClHA (34.0% of the 2-MCPD dose) shows that 2-ClHA is a major metabolite of 2-MCPD, whereas the structural isomer 3-ClLA represents only a minor amount of ingested 3-MCPD in human urine. For further considerations, we wanted to rule out the possibility that 2- and 3-MCPD (or pairs of their metabolites with the same atomic composition) may be formed by isomerization from each other or that the metabolites 2-ClHA and 3-ClLA may be formed by other substances in the hazelnut oil. Therefore, a male volunteer consumed the equivalent amounts of isolated 2- or 3-MCPD (present in 12 g hazelnut oil) in separate weeks. Data on the urinary analytes are summarized in Table S3, Supporting Information. In the 24 h after the intake of 2-MCPD or 3-MCPD, 659 nmol 2-ClHA or 18.7 nmol 3-ClLA were excreted, respectively, which was in the range observed in the study group on day 3 after the intake of 12 g of the hazelnut oil (Table 1 and 2). The same holds true for the daily "background" excretion of 2-ClHA (42 nmol) and of 3-ClLA (1.4 nmol) determined after the intake of 3-MCPD and 2-MCPD, respectively, compared to the background excretion in the study group. Taken together, the data supported the notion that 2-ClHA was not formed from 3-MCPD and that 3-ClLA was not formed from 2-MCPD. In addition, there was no indication that other

substances in the hazelnut oil may have contributed to the formation of 2-ClHA and 3-ClLA.

The discrepancy between the high amounts of 2-ClHA excretion and the low amounts of 3-ClLA excretion may be explicable if, for example, the enzymatic oxidation of 2-MCPD is more efficient compared to that of 3-MCPD (which may be primarily metabolized by other pathways), and/or if the turnover of 3-ClLA is more rapid compared to that of 2-ClHA. The latter notion is supported by previous data from animal experiments. The administration of [^{14}C]3-ClLA to rats led to the formation of radioactively labeled carbon dioxide and oxalic acid. The observation of the oxalic acid crystals in the urine of rats treated with 100 mg kg^{-1} bw 3-ClLA or 3-MCPD was accompanied by diuresis and enlargement of the kidneys, while higher doses of both compounds (120 to 150 mg kg^{-1} bw) led to anuresis and renal failure.[18] Noticeable changes of urinary oxalate levels were not expected in the current study because of its high daily "background" excretion, which was reported to be on average 28 ± 10 mg (403 women) and 39 ± 10 mg (414 men).[19] Although the mechanism is not known, the oxalic acid formation from 3-MCPD (spontaneous or involving enzymatic catalysis) would explain why 3-ClLA is not excreted in the urine in large quantities. Two other metabolic products of 3-ClLA were described in a cell-free system. Walsh et al. reported that 3-ClLA was degraded to pyruvate by lactate oxidase, a flavin-dependent monooxygenase isolated from *Mycobacterium smegmatis*, under anaerobic conditions, and to chloroacetate and carbon dioxide in the presence of oxygen.[20] However, the quantitative significance of these pathways in mammals is not known.

The metabolism of 2-MCPD has never been studied to the same extent. Formation of oxalate crystals and the associated nephrotoxic effect were not observed in male Sprague–Dawley rats following administration of 2-MCPD, even at doses of 200 mg kg^{-1} bw.[21] The current data support the hypothesis that 2-MCPD is oxidized efficiently to 2-chloro-3-hydroxypropionaldehyde by alcohol dehydrogenases followed by conversion to 2-ClHA by aldehyde dehydrogenases (Figure 1). The half-life time of 2-ClHA ($t_{1/2}$ = 8.2 h) is about twice as high as that observed for 3-ClLA, which indicated that 2-ClHA may be a final metabolite excreted after glomerular filtration without further metabolism.

Both 2-ClHA and 3-ClLA were considered as possible biomarkers of exposure to 2-MCPD and 3-MCPD, respectively. The metabolites were separated well by IPC and detected with high mass spectrometric sensitivity and specificity. In the case of 2-ClHA, the analytical method allowed a reliable quantification of urinary 2-ClHA excretion also for the "background" exposure. However, the specificity of 2-ClHA as a biomarker reflecting the uptake of 2-MCPD is questionable. The data of our study on the urinary excretion of 2- and 3-MCPD before and after the consumption of hazelnut oil allowed an estimation of 0.12 µg 2-MCPD/kg bw and 0.32 µg 3-MCPD/kg bw,[15] which is in line with the external exposure assessment of the EFSA.[3] In contrast, much higher estimates result if 2-ClHA and 3-ClLA are used for the calculation of the 2- and 3-MPCD external exposure. For 2-ClHA, the average ratio between the daily amounts of 2-MCPD excreted after and before the controlled exposure to the hazelnut oil in the study (33.7)[15] was much higher than that of the 2-ClHA excretion after and before exposure (12.9).

In case of 3-ClLA, several arguments contradict its use as a biomarker of exposure to 3-MCPD. First, the urinary excretion rate is low (mean: 0.28% of the 3-MCPD dose), and a part of the "background" samples were below the LOQ. Second, the interindividual variation of the urinary excretion of 3-ClLA after the exposure to the hazelnut oil was relatively high (CV = 52.9%), which limits the accuracy of single day exposure estimations for 3-MCPD in individuals using urinary 3-ClLA as a biomarker. And third—as observed in case of 2-ClHA and 2-MCPD - the estimate of the external "background" exposure to 3-MCPD from the daily excretion of 3-ClLA considerably exceeds the external estimate reported by the EFSA: the average ratio between the daily amounts of 3-MCPD excreted after and before the controlled exposure to the hazelnut oil in the study (27.8)[15] was much higher than that of the 3-ClLA excretion after and before exposure (5.4).

These discrepancies observed for 2-MCPD/2-ClHA and 3-MCPD/3-ClLA indicated a lack of specificity—the metabolites may not be formed exclusively from 2- or 3-MCPD, but also from other, hitherto unknown substances. There is little information on other possible sources. Early studies showed that propylene chlorohydrins, that is, 1-chloro-2-propanol and 2-chloro-1-propanol, were found in foods that had previously been treated with propylene oxide as fumigant for microbial sterilization.[22] In the rat, 1-chloro-2-propanol is further oxidized to 3-ClLA,[23] so that the formation of 2-ClHA from 2-chloro-1-propanol can be assumed. It is also possible that propylene oxide is chlorinated after direct absorption leading to the formation of propylene chlorohydrins in the stomach. There is no reliable data on human exposure to propylene oxide or propylene chlorohydrins.

In summary, this is the first report on two urinary metabolites in humans, 2-ClHA and 3-ClLA which are formed by oxidation of the heat-induced food contaminants 2- and 3-MCPD, respectively. The controlled exposure to a defined amount of bound 3-MCPD in form of a commercial hazelnut oil in a group of 12 adults led to the urinary excretion of only small amounts of 3-ClLA (mean: 0.28% of the 3-MCPD dose), probably due to other downstream metabolic pathways, for example, the conversion into oxalate, carbon dioxide, and chloride. In contrast, the high amount of urinary 2-ClHA indicated that this may be a final metabolite of 2-MCPD. Taken together all data on the human metabolism of fatty acid esters of 2-MCPD and 3-MCPD eaten in a serving of 12 g of hazelnut oil, we detected a mean of 48.3% of bound 2-MCPD (14.3% as 2-MCPD and 34.0% as 2-ClHA) and a mean of 4.01% of bound 3-MCPD (3.73% as 3-MCPD and 0.28% as 3-ClLA) in human urine.[15] The calculations of 2- and 3-MCPD external "background" exposure using 2-ClHA and 3-ClLA from the data in the current study led to overestimations, possibly because the metabolites are not as specific as required for such measurements of the internal exposure.

4. Experimental Section

Controlled Exposure Study: The human study was described in detail by Abraham et al.[15] In brief, the study group consisted of 12 healthy nonsmokers of European origin (six non-pregnant females and six males). Individual data are provided in Table S1, Supporting Information. The study was approved by the ethics committee of the Charité - Universitätsmedizin Berlin (No. EA4/130/17) and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later

amendments; it was registered in the German Clinical Trials Register (DRKS, No. 00013219). All participants got a detailed oral consultation about the rationale of the study and gave informed consent in writing. The study was conducted between October 2017 and January 2018 at the Department of Food Safety of the German Federal Institute for Risk Assessment (BfR). Urine samples were collected completely on four consecutive days (daily fractions 4 – 4 – 4 – 12 h, 1 h fractions during the first 8 h after the controlled exposure on day 3). The schedule of the study is outlined in Figure S1, Supporting Information. On Wednesday morning (day 3), the 12 participants ate 12 g of a 2-/3-MCPD ester-rich hazelnut oil (2-MCPD 24.2 mg kg^{-1}, 3-MCPD 54.5 mg kg^{-1}) containing doses of 290 µg 2-MCPD (2627 nmol) and 654 µg 3-MCPD (5917 nmol). The contents of the fatty acid esters of 2- and 3-MCPD were determined by the SGS Germany (Hamburg, Germany) according to a modification of the official method Cd 29b-13 ("3-in-1 method") of the American Oil Chemists' Society.[24]

The specificity of the metabolic conversion from 2-MCPD to 2-ClHA and from 3-MCPD to 3-ClLA was investigated by the uptake of 290 µg isolated 2-MCPD or 654 µg isolated 3-MCPD (equivalent to the amounts in 12 g of the hazelnut oil) by the medical head of the study (K.A.) in separate weeks. The portions of 2- and 3-MCPD were prepared from solutions with concentrations of 1 mg mL^{-1} in ethanol and were taken up together with 100 mL water. Urine was collected before exposure and in the time intervals 0–2 h, 2–4 h, 4–6 h, 6–8 h, 8–12 h, and 12–24 h thereafter.

Chemicals: High performance liquid chromatography (HPLC)-grade methanol, acetonitrile, and ethyl acetate were obtained from Merck (Karlsruhe, Germany). HPLC-grade water was prepared using a Milli-Q Integral Water Purification System from Millipore Merck (Darmstadt, Germany). 3-ClLA (≥92.5%), 2-ClHA (95%), acetic acid and aqueous hydrochloric acid (1 N), tributylamine, and other reagents were purchased from Sigma-Aldrich (Steinheim, Germany). The compounds [$^{13}C_3$]3-ClLA ([$^{13}C_3$]3-ClLA) and [$^{13}C_3$]2-ClHA ([$^{13}C_3$]2-ClHA) were synthesized by the ASCA GmbH (Berlin, Germany).

Liquid–Liquid Extraction of Urinary 2-ClHA and 3-ClLA: Aliquots of 1 mL urine were transferred into 5 mL centrifugation tubes and 50 µL aqueous hydrochloric acid (1 N) was used to acidify the urine to about pH 2. Then, 50 µL of the isotope-labeled internal standard solution containing 784 nM of [$^{13}C_3$]3-ClLA and 544 nM [$^{13}C_3$]2-ClHA dissolved in water/methanol (97:3) was added to each tube and vortexed for 1 min. A portion of 1.5 mL ethyl acetate was added and the mixtures were shaken for 10 min using a Reax 2 (Heidolph, Schwabach, Germany). After centrifugation at 3500 × *g* for 5 min, the extraction was repeated. The combined ethyl acetate fractions were washed with 1 mL of water (pH 2) acidified with aqueous hydrochloric acid (1 N) saturated with ethyl acetate. The supernatants were evaporated to dryness under reduced pressure. The dried samples were reconstituted in 100 µL of water/methanol (97:3), centrifuged at 12 000 × *g* for 5 min, and transferred to HPLC vials for LC-MS/MS analysis.

LC-MS/MS Analysis of 2-ClHA and 3-ClLA: Initial screening experiments aiming at the detection of chloride-abstracting compounds in human urine samples were done with an ultra HPLC system consisting of an Acquity I-Class (Waters, Eschborn, Germany) connected to a triple quadrupole-hybrid ion trap mass spectrometer QTrap6500 (Sciex, Darmstadt, Germany) equipped with an electrospray ionization source operating in the negative mode (System I). Chromatographic separation was carried out using an Acquity HSS T3 column (2.1 × 150 mm; 1.8 µm; Waters) at room temperature. The eluents were water containing 10 mM acetic acid (solvent A) and methanol/acetonitrile (95/5) (solvent B). An isocratic program was applied at a flow rate of 0.4 mL min^{-1} as follows: 0–6 min, 3% solvent B; 6–8 min, 100% solvent B; 8–10 min, 3% solvent B. The operating parameters of the QTrap6500 were: ion spray voltage 4500 V, interface heater temperature 450 °C, curtain gas 40 psi, ion source gas 1 60 psi, ion source gas 2 50 psi, collision activated dissociation gas set to medium. The multiple reaction monitoring (MRM) mode was employed for quantitative analysis with the declustering potential and the entrance potential at −47 and −10 V, respectively. The fragmentation-specific values for collision energies and cell exit potentials of 2-ClHA, 3-ClLA, and their isotope-labeled standards are summarized in Table S2, Supporting Information. Data acquisition and processing were carried out using Analyst 1.6.2 software (Sciex).

An IPC-method with superior chromatographic separation (System II) was used for the quantification of 2-ClHA and 3-ClLA consisting of an HPLC 1100 (Agilent, Waldbronn, Germany) equipped with a Nucleoshell RP 18plus (2.0 × 150 mm, 2.7 µm; Macherey–Nagel, Düren, Germany) connected to a triple quadrupole-hybrid ion trap mass spectrometer QTrap6500 (Sciex). The eluents were water containing 10 mM tributylamine and 10 mM acetic acid (solvent A) and acetonitrile (solvent B). The flow rate of the gradient (0–3 min, 2% solvent B; 3–6.5 min, 2–36% solvent B; 6.5–8 min, 36–95% solvent B; 8–10.5 min, 95% solvent B; 10.5–15 min, 2% solvent B) was 0.5 mL min^{-1}. The temperature of the column oven was set to 40 °C and the sample injection volume was 8 µL. The mass spectrometric parameters were as described above.

Validation of the Analytical Quantification of 2-ClHA and 3-ClLA: The interference of [$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA with the MRM traces of the analytes was assessed by injecting 8 µL of a solution containing [$^{13}C_3$]2-ClHA (2722 nM) and [$^{13}C_3$]3-ClLA (3922 nM). Afterward, blank samples were injected to determine the carryover. The linearity of detection was determined using solutions of the isotope-labeled standards ([$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA) because of the presence of 2-ClHA and 3-ClLA in human urine samples. The effect of the sample matrix on the mass spectrometric detection of the analytes was assessed by comparing the slopes of the calibration lines of [$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA determined in water/methanol (97:3) with those determined in the presence of extracts of pooled urine samples (matrix). The LOD (signal-to-noise ratio (S/N) > 3) and LOQ (S/N > 10) were determined from the calibration lines of [$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA, prepared with or without the urinary matrix.

The overall recoveries of the extraction with ethyl acetate were determined by processing 1 mL of pooled urine spiked at three different concentrations of [$^{13}C_3$]2-ClHA (10.9, 54.4, or 272 nM) and [$^{13}C_3$]3-ClLA (15.7, 78.4, or 392 nM) as described above. The recovery was calculated by comparing the average peak area of [$^{13}C_3$]2-ClHA and [$^{13}C_3$]3-ClLA in the samples with those determined after dissolving the expected analyte amounts in the presence of extracts from unspiked urine samples. The precision was determined by analyzing urine samples spiked with three different concentrations of 2-ClHA or 3-ClLA (8.03, 80.3, and 803 nM, intraday precision $n = 5$; intraday precision $n = 6$).

High-Resolution Mass Spectrometry of 2-ClHA: A pool urine sample of five subjects collected 8–12 h after the consumption of 12 g hazelnut oil was extracted as described. In order to increase the sensitivity of the analysis, 2 mL urine (instead of 1 mL) was extracted and reconstituted in 100 µL water/methanol (97:3). The determination of the exact mass and the recording of the MS^2 fragment ion spectra of 2-ClHA were done on an Acquity I-Class UPLC (Waters) connected to a TripleTOF 6600 (Sciex) equipped with an electrospray ionization source operating in the negative mode. Chromatographic separation was carried out using an Acquity HSS T3 column (2.1 × 150 mm; 1.8 µm; Waters) at 40 °C. The injection volume was 10 µL. The eluents were water containing 10 mM acetic acid (solvent A) and methanol/acetonitrile (95/5, solvent B). A gradient program was applied at a flow rate of 0.3 mL min^{-1} as follows: 0–2 min, 3% solvent B; 2–20 min, 3–40% solvent B; 20–20.1 min, 40–95% solvent B; 20.1–23 min, 95% solvent B; 23–24 min, 95–3% solvent B; 24–25 min, 3% solvent B. The operating parameters of the TripleTOF 6600 were: ion spray voltage 4500 V, interface heater temperature 450 °C, curtain gas 55 psi, ion source gas 1 60 psi, ion source gas 2 70 psi. The exact mass of 2-ClHA was determined by a time of flight (TOF)-MS scan from 34 Da to 1200 Da. Simultaneously, the MS^2 fragment ion spectrum was recorded by a product ion scan of the ion with $m/z = 123$ Da using a declustering potential of −30 V and a collision energy of −25 V (spread 15 V) was used. Data acquisition and processing were carried out using Analyst 1.7.1 software (Sciex).

Statistical Evaluation: The 3-ClLA concentration of one sample below the LOD (S/N < 3; 1 out of 264) was set to LOD/2 (0.2 nM 3-ClLA in the urine) for the evaluation. The interval censored data on 3-ClLA concentrations (3 < S/N < 10; 47 out of 264) were used as such, because this

approach ensures a higher statistical reliability compared to the replacement of all analytical results with LOQ/2.[25] The 2-ClHA signals were all above the LOQ (S/N > 10). Standard calculations were done with Excel (Microsoft Office 2016).

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.A. designed the human study, obtained the ethical approval, and was responsible for the information of the participants. N.B. and Z.Z. contributed equally in developing the analytical methods and performing experimental analyses. The analytical work was supervised by B.H.M., who compiled and analyzed the data. All authors contributed to the writing of the manuscript.

Data Availability Statement

Data available on request due to privacy/ethical restrictions.

Keywords

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