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Determination of sorbic acid in urine by gas chromatography–mass spectrometry

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Abstract

The average daily uptake of the common food preservative sorbic acid is estimated to range from 0.01 to 1.1 mg kg^{-1}. Sorbic acid mainly is metabolised to carbon dioxide. Minor amounts are converted to *trans,trans*-muconic acid (ttMA) as well as excreted unchanged into the urine. Since urinary ttMA is a biomarker for the occupational and environmental exposure to benzene, there is an additional need for monitoring the uptake of sorbic acid, particularly at low, environmental benzene exposure levels. For this purpose, a simple, robust and rapid method for the determination of sorbic acid in urine at trace levels was developed. After addition of 10 ml of water and 5 ml of 8 *M* hydrochloric acid to 10 ml of the thawed urine, the sample was water steam distilled using an automated distillation device. A total of 100 ml of the distillate were solid-phase extracted. After washing, the sorbic acid was eluted with 4 ml methanol. The eluate was reduced under a stream of nitrogen to a volume of 300 μl. After addition of 500 μl boron trifluoride in methanol and incubation for 1 h at 60°C, the resulting sorbic acid methyl ester was extracted three times with 1 ml heptane. To the combined heptane layers, sorbic acid ethyl ester was added as an internal standard. After reducing to a volume of 100 μl in a stream of nitrogen, the final analysis was performed by GC–MS using the fragment ions m/z 126 for the analyte and m/z 140 for the internal standard. The limit of detection was 0.7 ng ml^{-1} urine and the R.S.D. of 69 duplicate determinations was 7.5%. In a controlled, experimental study and in a field study, we were able to show that urinary sorbic acid is a marker for the dietary uptake of sorbic acid and that sorbic acid is converted to ttMA. On average, 0.1% of the dietary sorbic acid is excreted unchanged into the urine. Excretion is complete within 24 h. We found that, on average, 0.23% of the oral dose of sorbic acid is excreted as urinary ttMA. There was a significant correlation between urinary excretions of sorbic acid and ttMA ($r=0.74$, $n=69$). We conclude that urinary sorbic acid can be used to correct the urinary ttMA level in order to determine the portion related to benzene exposure. This appears to be necessary particularly at low, environmental benzene levels.

Keywords: Food analysis; Sorbic acid; Muconic acid

1. Introduction

During the last decades sorbic acid (*trans,trans*-2,4-hexadienoic acid, E200) and its potassium salt (E202) have been accepted as 'generally recognized as safe' (GRAS) substances [1] and have become the leading preservatives for food [2] as well as for pharmaceutical and cosmetic preparations. Neither sodium nor calcium sorbate appeared to be used in foods [3].

The anti-fungous activity of sorbic acid ($pK_a=$

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4.75) and its salts increases with the concentration of the undissociated acid at low pH values [4]. The acceptable daily intake (ADI) of sorbate (expressed as sorbic acid) is 25 mg kg^{-1} body mass [5]. The sources for the intake of sorbate are bread and pastries, fats and oils, cheese, meat products (e.g., bacon, sausages), tinned food, processed vegetables and juices, candies, jams, wine and beverages. The average daily per capita intake of sorbic acid was estimated to be up to 5 mg kg^{-1} [6]. Van Dokkum et al. verified a daily intake of 0.1-−0.4 mg kg^{-1} with fats and oils as the main sources for sorbic acid [7]. Except for these rough estimates, no reliable data on the uptake of sorbic acid are available.

Trans,trans-muconic acid (ttMA) is used as a biomarker for high, occupational and low environmental exposure to benzene [8,9]. Westöö et al. [10] observed that mice convert 0.2–0.6% of the ingested [1-^{14}C]sorbic acid to urinary muconic acid. In humans, the conversion of sorbic acid to ttMA is reported to be 0.13–0.18 [11], 0.08–0.19 [12] or 0.21–0.34% [13]. Assuming a daily intake of approximately 30 mg [7], it is estimated that sorbic acid would account for at least 50% of the ttMA background urinary excretion in non-occupationally exposed non-smokers and 25% in smokers [12]. Pezzagno and Maestri [13] even assumed a daily ingestion of 300–500 mg sorbic acid, which would lead to an excretion of 1 mg ttMA per day, corresponding with an exposure to 1 ppm benzene over 8 h. The available data suggest that the common ingestion of sorbic acid significantly contributes to the total urinary excretion of ttMA of non-occupationally exposed individuals. In order to further quantify this contribution, we developed a method for the determination of sorbic acid in human urine as a marker for its dietary uptake. Furthermore, we examined the possibility to correct urinary ttMA levels for the contribution of sorbic acid.

2. Experimental

2.1. Chemicals

Sorbic acid (>99%; order No. 85510) and hydrochloric acid (37%; order No. 84426) were purchased from Fluka (Buchs, Switzerland), sorbic acid ethyl ester (98%; order No. 17.768-7) was from Aldrich (Steinheim, Germany), acetonitrile (gradient grade; order No. 1.00030.9010), methanol (gradient grade; order No. 1.06007.9010) and boron trifluoride (20% in methanol; order No. 8.01663.0100) were from Merck (Darmstadt, Germany). Diethyl ether (order No. 9134), 2-propanol (order No. 4359), ethanol (order No. 9812) and heptane (order No. 9073) were purchased from Promochem (Wesel, Germany). The test kit for creatinine (Merckotest, order No. 1.03385.0001) was purchased from Merck.

2.2. Instrumentation

All glassware were cleaned with acetone and heptane and heated for 2 h at 150°C prior to use. The automated steam distillation device (VapoDest 20) was purchased from Gerhardt (Bonn, Germany). Solid-phase enrichment was performed with a vacuum-extraction manifold (210224; Alltech, Deerfield, IL, USA) with a drying attachment (212124), using disposal cartridges with 200 mg LiChrolut EN (1.19870.0001, Merck). GC–MS was performed using a gas chromatograph (GC 8000^{TOP}) equipped with an automated sampler (AS 800), a split/splitless injector (SSL71) and a mass detector (MD 800). All devices were purchased from ThermoQuest (Egelsbach, Germany). A SGE BPX5 column (60 m×0.25 mm, 0.25 μm, AZ Poeck, Langerwehe, Germany) was applied for gas chromatographic separation.

For the determination of creatinine a multi-channel photometer (SLT, Spektra, Strasbourg, France) and 96-well microplates (polystyrol, Greiner, Frikkenhausen, Germany) were applied.

2.3. Study design

2.3.1. Study 1 (field study)

Sixty-nine free-living, healthy volunteers (23 smokers, 46 non-smokers, aged 18–70) collected 24-h urine samples in polyethylene bottles. The samples were stored at +4°C during sampling and afterwards at −25°C until analysis of sorbic acid, ttMA and creatinine. No special diet was applied.

2.3.2. Study 2 (experimental study)

Seven healthy volunteers (three smokers, four

non-smokers, aged 28–48) collected 8-h urine samples on each of the five study days starting at 07:00 h. During Days 2 and 3, the volunteers ingested three daily doses of sorbic acid (1 mg kg^{-1} body mass dissolved in water) at breakfast (08:00 h), lunch (12:00 h) and dinner (18:00 h). All subjects ate an identical diet, low in sorbic acid, over the whole study period to insure a constant background intake of sorbic acid during the study. Urine samples were collected in 2-l polyethylene bottles containing 2 ml 8 *M* hydrochloric acid and kept at +4°C during sampling. The urine samples were stored frozen (−25°C) until analysis.

2.4. Methods

Ten ml of a freshly thawed urine were combined with 10 ml water and 5 ml 8 *M* hydrochloric acid. The solution was water steam distilled (VapoDest 20, 100% efficiency) for 210 s. A distillate of 100 ml was collected in a glass flask containing 500 μl 8 *M* hydrochloric acid. The distillate can be stored for up to 1 week in the refrigerator. The total distillate was applied on a solid-phase extraction (SPE) (LiChrolut EN) cartridge, which was conditioned with acetonitrile, methanol and 40 m*M* hydrochloric acid (each 3×3 ml), prior to use. The cartridge was washed with 2 ml each of 40 m*M* hydrochloric acid and water, and dried in a stream of nitrogen for 30 min. After elution of the sorbic acid with 4×1 ml of methanol, the eluate was reduced to a volume of approximate 300 μl in a stream of nitrogen. The residue was incubated with 500 μl of boron trifluoride in methanol for 1 h at 60°C in the dark. To the resulting solution of the methyl ester, 100 μl of sorbic acid ethyl ester in diethyl ether (10 $\mu g\ ml^{-1}$) were added as an internal standard. The sorbic acid esters were extracted three times with 1 ml of heptane. The heptane layers were combined and reduced to 100 μl by applying a stream of nitrogen. Two μl of this concentrate were injected into the GC–MS system. The initial temperature of 75°C (initial time 1 min) was raised with 10°C min^{-1} to 285°C (final time 4 min). Helium was used as carrier gas with a constant flow of 0.6 ml min^{-1}. The injector temperature was 260°C. The injection was splitless with split on (50 ml min^{-1}) at 1 min. The total run time was 26 min. The transfer line to the MS was hold at 280°C. The mass traces 126 m/z and 140 m/z were recorded in the single ion recording mode. The retention times under these conditions were 12.65 and 13.96 min for sorbic acid methyl ester and sorbic acid ethyl ester, respectively. Quantitation was performed using the ratio of the peak areas of sorbic acid methyl ester (126 m/z) and sorbic acid ethyl ester (140 m/z). A 15-point calibration curve over a range of 10–500 ng ml^{-1} was constructed with spiked human urine samples.

The method for the determination of the ttMA has been described elsewhere [12,14].

Creatinine was determined photometrically using the Jaffé reaction by applying a test kit (Merck) [15,16].

3. Results

Fig. 1 shows a chromatogram of a human urine sample with a sorbic acid concentration of 101 ng ml^{-1}. By water steam distillation, sorbic acid is fairly well separated from the urinary matrix. Sorbic acid was detectable in all samples investigated. The limit of detection (LOD) was 0.7 ng ml^{-1} when applying the three-times signal-to-noise ratio procedure. The limit of quantitation (LOQ) was 1.3 ng ml^{-1} when applying the 10-fold signal-to-noise ratio procedure. The coefficient of variation was 18.6% for 10 repetitions of a sample with a concentration level of 2 ng ml^{-1} (intra-assay precision). The mean inter-assay precision for duplicates of 69 samples was 7.5%. The method proved to be linear in the range of 1.5–1500 ng ml^{-1}. The recovery of sorbic acid after water steam distillation was 91%. The recovery rate of the analyte for the total procedure was 85%. The sample throughput is about 24 samples per day when using a 24-port manifold for the SPE.

The average daily urinary excretion of sorbic acid of 67 subjects during the field study was found to be 0.42 μg kg^{-1} (range, 0.12–1.03 μg kg^{-1} per day). In the experimental study with low dietary intake of sorbic acid, the mean background excretion of seven subjects was 0.24 μg kg^{-1} (range, 0.18–0.34 mg kg^{-1} per day). Fig. 2 shows the time course of the urinary excretion of sorbic acid over the 5-day study

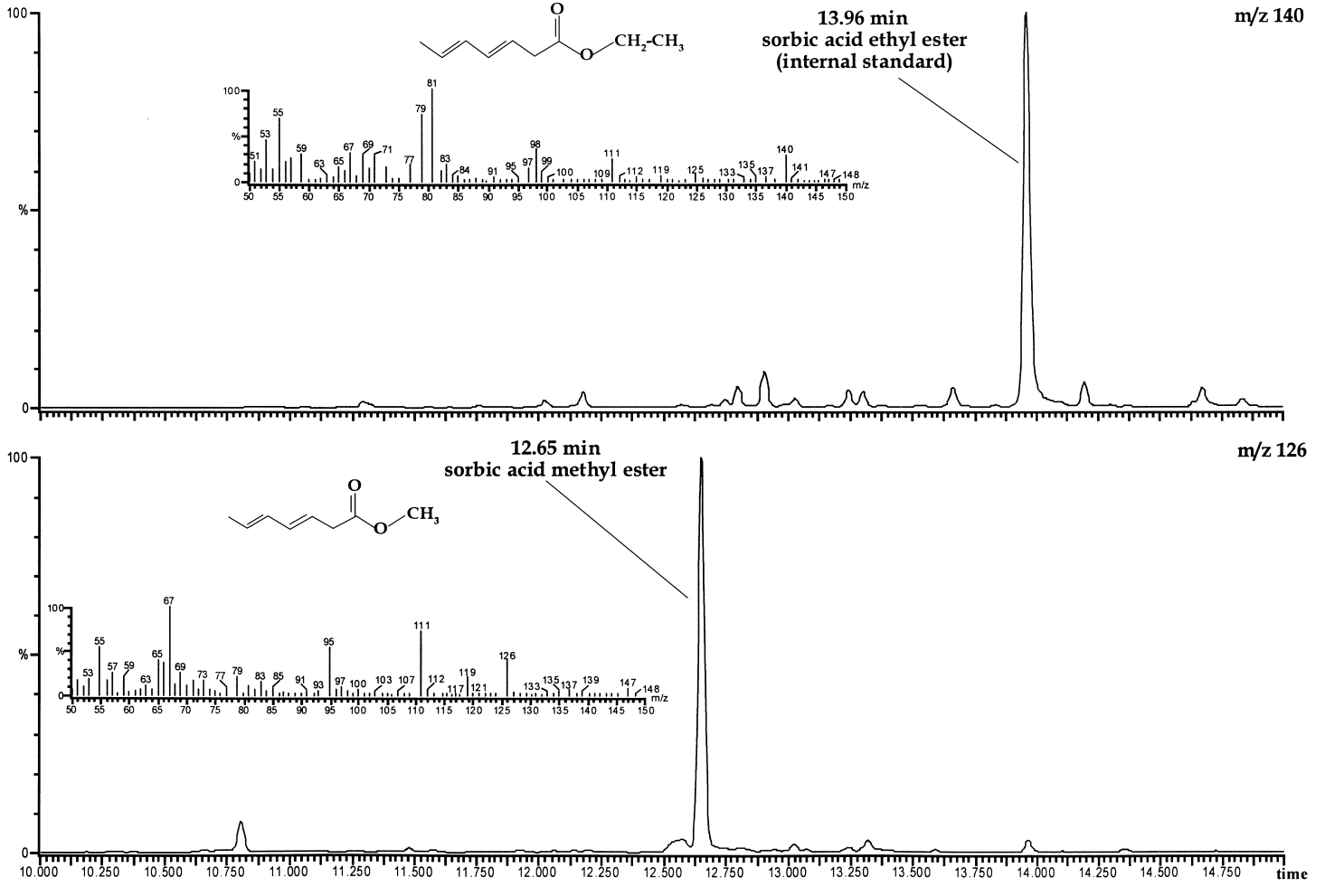

Fig. 1. Chromatograms (single ion recording, 126 and 140 m/z) and spectra of sorbic acid methyl ester (12.65 min) and sorbic acid ethyl ester (13.96 min, internal standard) of a urine sample (101 ng ml^{-1}) after sample preparation.

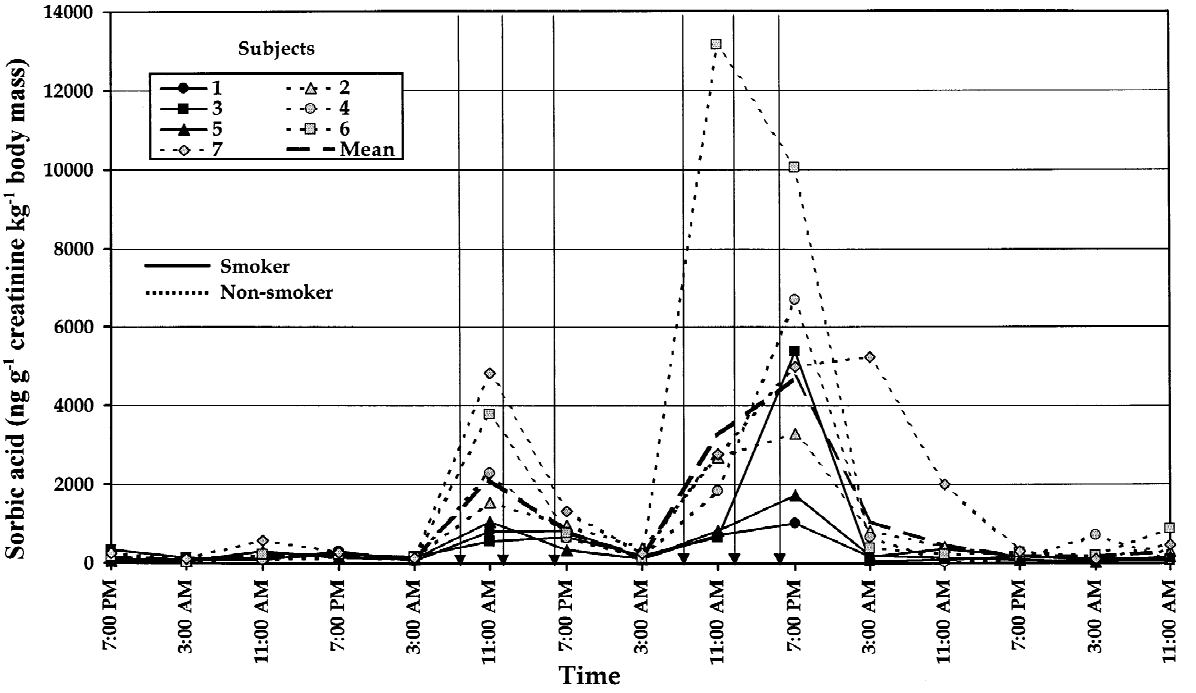

Fig. 2. Time course of urinary sorbic acid excretion over 5 days in Study 2. Sorbic acid was given as oral application in three doses (1 mg kg^{-1} body mass, dissolved in water) each on Days 2 and 3. Times of application are indicated as arrows in the figure.

period. After oral application of 1 mg kg^{-1} body mass the excretion of sorbic acid increased in all participants, although to a different extent. After the first day with sorbic acid application, the urinary excretion decreased to almost background levels and increased to even higher levels on the second day of sorbic acid application. Urinary excretion of sorbic acid after oral application was found to be complete within 24 h. The half-life of ingested sorbic acid is estimated to be less than 8 h. The percentage of unchanged sorbic acid excreted into the urine was found to be 0.095% (range, 0.02–0.19%).

In Fig. 3, the time course of urinary excretion of ttMA after oral application of sorbic acid is shown. The urinary elimination kinetics of ttMA is similar to that of sorbic acid. However, the average amount of ttMA excreted after ingestion of experimental doses of sorbic acid are similar on both days. The conversion of sorbic acid to urinary ttMA was found to be 0.23% (range, 0.15–0.34%). The background level for smokers is slightly higher than for non-smokers. Non-smokers showed higher amounts of urinary sorbic acid and of ttMA on the second day of sorbic acid application than smokers.

In the field study, a significant correlation between the urinary excretion of sorbic acid and ttMA was found ($r=0.74$, $n=69$; Fig. 4). Excluding the two subjects with the highest urinary excretion of ttMA (>700 μg l^{-1}) still leads to a significant correlation ($r=0.65$, $n=67$). It is interesting to note that one of these subjects also exhibited the highest urinary sorbic acid concentration (~350 μg l^{-1}) in our field study (Fig. 4). With the conversion factor derived from the experimental study (0.095%), an average daily uptake of sorbic acid of 0.44 mg kg^{-1} can be calculated. This amount of sorbic acid can give rise to 1.17 μg kg^{-1} per day of ttMA in urine (range, 0.29–2.49 μg kg^{-1} per day).

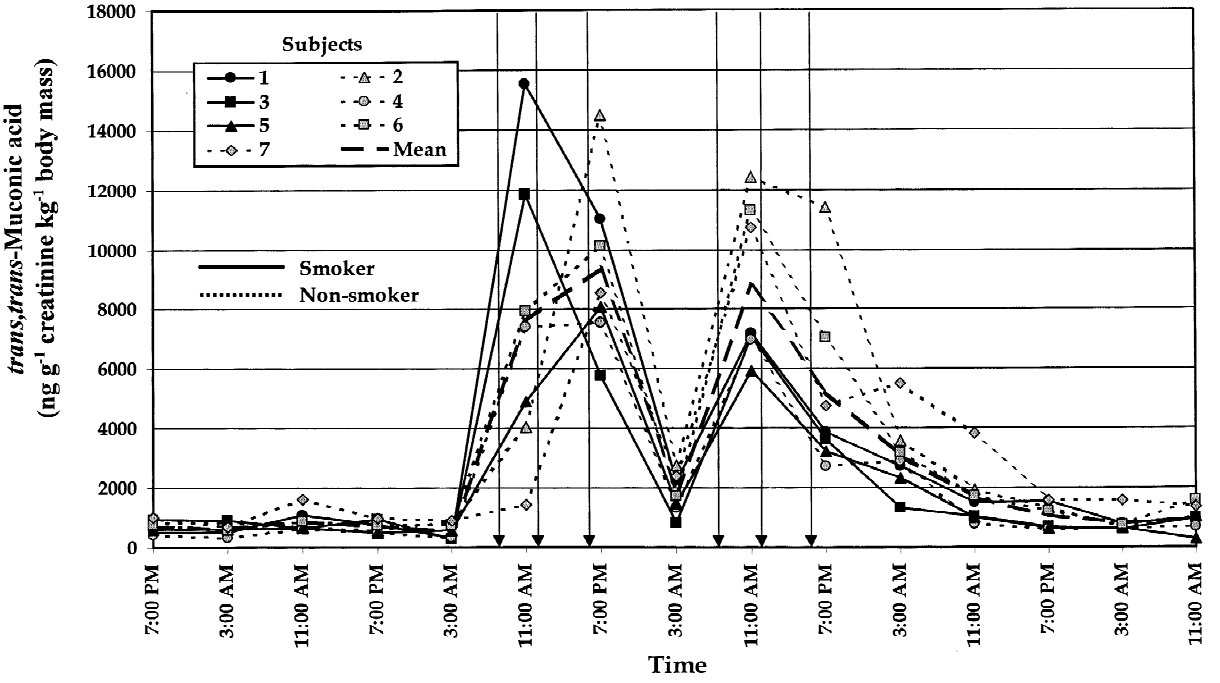

Fig. 3. Time course of urinary ttMA excretion over 5 days in Study 2. Sorbic acid was given as oral application in three doses (1 mg kg^{-1} body mass, dissolved in water) each on Days 2 and 3. Times of application are indicated as arrows in the figure.

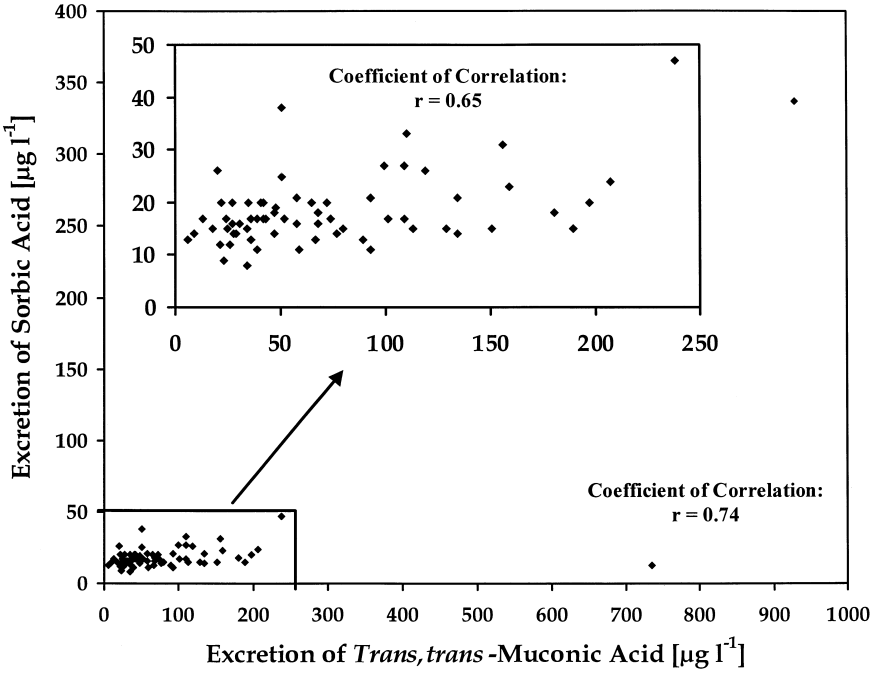

Fig. 4. Correlation between urinary concentrations of sorbic acid and ttMA in 24-h urine samples of 69 healthy volunteers with normal diet (Study 1).

Table 1
Averages (ranges) of urinary excretion of sorbic acid and ttMA, calculated daily uptake of sorbic acid and amount of urinary ttMA derived from dietary sorbic acid

	Urinary excretion of sorbic acid ($\mu g\ kg^{-1}$ per day)	Urinary excretion of ttMA ($\mu g\ kg^{-1}$ per day)	Calculated average daily intake of sorbic acid ($mg\ kg^{-1}$ per day)	Percentage of urinary ttMA derived from dietary sorbic acid (%)
Study 1 ($n=67$)	0.42 (0.12–1.03)	2.22 (0.32–5.98)	0.44 (0.16–1.08)	53 (4–525)
Study 2 ($n=7$)	0.24 (0.18–0.34)	1.08 (0.78–1.28)	0.37 (0.09–0.77)	59 (24–86)[a]

[a]For $n=6$; one subject was excluded from the evaluation because of a very low excretion of unchanged sorbic acid leading to an unrealistically high intake of sorbic acid and percentage contribution to urinary ttMA (180%).

4. Discussion

Table 1 summarises the data on urinary excretion of sorbic acid and ttMA after ingestion of sorbic acid, as well as the calculated amounts of sorbic acid ingested and the percentage of ttMA derived from sorbic acid. Calculations of the data of Study 1 were performed using the average conversion factors determined in Study 2, whereas the results of Study 2 were calculated with the individual factors.

From Study 1, we estimate that the average daily uptake of sorbic acid is about 0.4 mg kg^{-1} (range, 0.1–1 mg kg^{-1} per day). We observed a high inter-individual variation (almost 10-fold) in the metabolism and urinary excretion of sorbic acid. On average, 0.1% of the ingested amount of sorbic acid is excreted unchanged into the urine and 0.23% appears as ttMA in the urine. To our knowledge, it is the first time that the amount of the dietary sorbic acid excreted unchanged into the urine was determined in humans at normal dietary levels. This percentage is significantly lower than that reported by Westöö [10], who found that 0.7% of sorbic acid fed to mice were excreted unchanged into the urine.

The mean conversion rate of sorbic acid ingested to urinary ttMA was found to be 0.23% and thus confirms earlier results [11–13]. With the results obtained in Study 2, a combined conversion factor (urinary sorbic acid to urinary ttMA) of 2.45 (range, 1.13–10.8) can be calculated. This factor would allow to estimate the contribution of dietary sorbic acid to the urinary ttMA. Our results show that this contribution is significant (53–59%) in subjects exposed to environmental and smoking levels of benzene. Unfortunately, the inter-individual variations in the metabolism and renal elimination of both sorbic acid and ttMA are large so that for a part of the subjects no meaningful contribution rates ($>>$ 100%) were obtained in Study 1, when an average conversion factor was used (Table 1). However, we believe that the correction of the confounding effect of sorbic acid on urinary ttMA level is useful in subjects with uncommonly high intake of sorbic acid and low, environmental benzene exposure.

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