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ORIGINAL ARTICLE

Ethyl glucuronide and ethyl sulfate in urine after consumption of various beverages and foods—misleading results?

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Abstract Urine testing for ethyl glucuronide (EtG) is used to spot recent alcohol intake and is utilized to document alcohol abstinence. However, other possible sources of ethanol existed when special beverages or foods were ingested. EtG concentration curves in urine were measured after the consumption of non-alcoholic beers, fruit juices, sauerkraut, and matured bananas. Using a cutoff of 0.1 mg/l, positive EtG findings were revealed after the ingestion of a lot of non-alcoholic beer up to 13 h later, sauerkraut up to 5 h later, and matured bananas up to 3.5 h later. In German abstinence programs, subjects have to deliver a urine sample within 24 h after advice, and all participants are informed about possible misleading results caused by the consumption of certain beverages or foods. With respect to the present results, a 0.1 mg/l cutoff can be considered useful, and misleading results should not be expected from informed subjects within a 24-h waiting period.

Keywords Ethyl glucuronide (EtG) · Ethyl sulfate (EtS) · Urine · Abstinence · Alcohol · Food · LC/MS

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Introduction

Ethyl glucuronide (EtG) and ethyl sulfate (EtS), which are non-volatile and water-soluble metabolites of ethanol, are formed by the conjugation of ethanol with UDP-glucuronic acid or activated sulfate via the action of UDP-glucuronosyltransferase (UDP-GT) and sulfotransferases, respectively [1–7]. Only a small amount of the ethanol ingested (<0.1%) becomes conjugated to these metabolites, which are specific for the presence of ethanol. After the consumption of alcohol, EtG and EtS are excreted for considerably longer times than the ethanol. Therefore, urine testing for these minor ethanol metabolites has gained popularity as a sensitive method to spot recent alcohol intake in clinical and forensic investigations and is utilized to document alcohol abstinence.

EtG and EtS are detectable in urine samples for ≤24 h after the intake of ≤0.25 g ethanol per kilogram body weight and for ≤48 h after the intake of 0.50 g/kg [4, 8–12]. After recovering from heavy drinking, the detection windows for EtG and EtS in alcohol-dependent patients ranged from <24 h to >90 h up to 130 h [13–15].

Urine EtG concentrations are influenced by diuresis and thus correlated with urine creatinine [8, 16, 17]. As such, urinary EtG results below a cutoff level can be achieved in samples with low urine creatinine concentrations if internal dilution is not taken into account. To avoid such false-negative findings, a correction of the urine EtG concentration for urine creatinine is recommended before interpretation. This is typically accomplished by reporting a normalized EtG result to a typical urine creatinine concentration (e.g., 100 mg/dl), designated as EtG_{100}, or by rejecting samples in which the creatinine concentration is below a critical value, such as 20 mg/dl.

Various analytical cutoff limits (0.05–1 mg/l) are used for the determination of EtG in urine samples. A threshold of 0.5 mg/l EtG in urine was proposed to obtain a high sensitivity (positive findings that are correctly identified in cases of intentional alcohol consumption), but it avoids positive results due to unintentional ethanol exposure, which is normally given by incorporation of lower doses [18, 19]. Recently, in Germany, new guidelines for driving ability diagnostics were recommended. For the control of alcohol abstinence, persons involved have to pass several urine tests after being summoned without prior notice. Within 24 h after receiving a summons, they have to deliver a urine sample under visual control. Samples with a creatinine concentration below a critical value of 20 mg/dl should be rejected. To extend the window of detection in such abstinence programs, a cutoff value of 0.1 mg/l EtG is used, but all participants were elucidated concerning the risk of misleading results due to items that contain ethanol, such as foods, beverages, medications, or cosmetics/sanitizers. However, in single cases after positive results, alcohol consumption is negated and competing causes are declaimed.

The aim of the present study was the evaluation of other possible sources of ethanol and therefore for positive EtG and EtS results typically discussed in abstinence programs.

Materials and methods

Chemicals

EtG, EtS, d_5-EtG, and d_5-EtS were purchased from Lipomed (Arlesheim, Switzerland). Methanol, ethanol, *tert*-butanol (internal standard for ethanol analysis), acetonitrile, and formic acid (98%) were obtained from Merck (Darmstadt, Germany). All chemicals were of the highest analytic grade. Water was purified with a NANOpure Diamond Analytic Water Purification System D11901 (Barnstead, Dubuque, IA, USA).

Stock solutions of EtG/EtS (1 g/l) and d_5-EtG/d_5-EtS (5 g/l) were prepared in methanol by weighing them separately, and they were stored at −20°C. Working standard solutions used for calibration were prepared by spiking blank urine at 0.025, 0.05, 0.1, 0.25, 0.5, 1, and 2 mg/l. Quality control samples were prepared at 0.1, 0.75, and 1.5 mg/l. All working solutions were stored in a refrigerator (2–8°C) when not in use.

Alcohol analysis of various beverages

Various beverages and foods were tested for the presence of ethanol using a routine headspace gas chromatographic procedure with a flame ionization detector (HS-GC/FID) [20]. Fruit juices were analyzed directly after opening and additionally 7 days later after storage at 4°C in a refrigerator or at room temperature to investigate potential alcohol formation due to fermentation.

Sample preparation for EtG/EtS analysis

To 100-µl urine samples, 20 µl of the internal standard (d_5-EtG and d_5-EtS 2.5 µg/ml in methanol) and 380 µl of methanol were added for protein precipitation. The samples were centrifuged for 10 min at 3.000×*g*. The supernatant was separated and evaporated to dryness under a stream of nitrogen at 40°C. The dried extracts were reconstituted with 600 µl of 0.1% aqueous formic acid, and 10-µl aliquots were directly injected into the liquid chromatographic–tandem mass spectrometric (LC–MS/MS) system.

LC–MS/MS analysis

Analyses were performed on a Shimadzu LC-20A Series system (Shimadzu, Duisburg, Germany) interfaced to a 4000 Q-Trap (Applied Biosystems/Sciex, Darmstadt, Germany) with an electrospray Turbo V™ Ion (ESI) source in negative mode. The ESI source settings were ion-spray voltage of −4,500 V, source temperature of 450°C, and nebulation and heating gas (N_2) of 60 and 50, respectively. For chromatographic separation, a polar end-capped phenylpropyl reversed-phase column (Synergi Polar-RP 250 × 2 mm, 4 µm) with a guard column (ODS Octadecyl 4 mm × 2 mm; Phenomenex, Aschaffenburg, Germany) was used at 40°C. A mobile phase of water containing 0.1% of formic acid (solvent A) and acetonitrile (solvent B) was used with a flow rate of 0.1 ml/min and the following gradient program: 100% A for 6 min, on 100% B in 1 min for 2 min, and then back to 100% A in 1 min for 4 min. Acetonitrile was added post-column (0.2 ml/min) by a tee mixer to enhance analyte ionization. Detection of the ions was performed in the multiple reaction monitoring mode using the following precursor to product ion transitions: EtG 221/75 (target), 221/85, 221/113; d_5-EtG 226/85 (target), 226/75; EtS 125/97 (target), 125/80, 125/64; and d_5-EtS: 130/98 (target), 130/80. For quantification, peak area ratios of the analytes to the internal standard were calculated as a function of the concentration of the substances.

The LC–MS/MS procedure was validated according to the guidelines of the GTFCh [21]. The seven-point calibration curve was linear over the whole range. The average equations for EtG and EtS (n=6) were $y=2.98\times10^{-3}x+1.14\times10^{-2}$ and $y=1.44\times10^{-3}x+9.71\times10^{-3}$,

respectively, with mean correlation coefficients of 0.9989 and 0.9982, respectively. The limits of detection (LOD) of 0.005 mg/l for both analytes, as well as the limits of quantitation (LOQ) of 0.019 mg/l (EtG) and 0.015 mg/l (EtS), were calculated. Carryover was excluded by the analysis of blank samples between two authentic samples. Benchtop stability as well as ion suppression/enhancement was tested according to the GTFCh guidelines and was negligible. The accuracy and the precision of the method were assessed by analyzing two replications of QC samples at concentrations of 0.1, 0.35, and 1.5 mg/l on eight consecutive days. The method showed an accuracy within 10%. The intra- and inter-day precision (RSD) values for QC samples were always less than 5%.

Creatinine measurement

Creatinine in urine was determined via the Jaffé reaction by using the DRI® Creatinine-Detect® test (Microgenics, Passau, Germany) on a Hitachi 912 automatic analyzer according to the manufacturer's guidelines.

Experimental setup of the ingestion experiments

After at least 80 h of abstinence from alcoholic beverages and the collection of a void urine sample, five to eight volunteers (all classified as social drinkers) orally consumed the following beverages and foodstuffs:

1. Between 2.0 and 3.0 l of a so-called non-alcoholic beer (maximum 4 g ethanol per liter)
2. Between 1.1 and 2.0 l of apple juice (maximum 3 g ethanol per liter)
3. Between 1.5 and 2.0 l of grape juice (maximum 7.9 g ethanol per liter)
4. Between 750 and 1,320 g sauerkraut (minimum 2% wine)
5. Between 670 and 690 g of matured peeled bananas

All information is summarized in Table 1, including the personal data of the participants and doses ingested. The beverages were ingested, and within 1 to 2 h and up to 50 h, urine samples were collected from each participant. During the first 8 h after ingestion, samples were collected every 1 to 2 h. The participants were informed of the purpose of the study and gave their voluntary consent to take part in the experiments. There was no relationship of dependency between the participants and the responsible heads of the studies. All analytical results and personal information of the participants have been anonymized. Besides, the ingestion experiments involved the consumption of a prevalent beverage or foodstuff and so there is no extra risk for the participants. On this basis, no application for ethical review was required.

Table 1 Participants and ingested amounts

No.	Age (years)	♀/♂	Weight (kg)	Height (cm)	Beer (non-alcoholic) (l)	Sauerkraut (g)	Apple juice (l)	Grape juice (l)	Bananas (peeled) (g)
1	19	f	75		2.3				
2	26	f	75	177	2.5	850	2.0	1.7 (red)	690
3	23	f	77		2.0				
4	24	f	77	175	2.5	750			
5	29	f	55	172	2.5				690
6	23	f	64	169	2.0	750	2.0		
7	26	m	63		3.0				
8	26	f	65		2.0				
9	28	m	86	185		1,320			
10	27	f	78	168		750			
11	22	f	56	163			2.0		
12	26	m	78	188			2.0	2.0 (white)	690
13	24	f	52	160			1.1		690
14	29	f	68	160				1.5 (red)	
15	27	f	48	156				1.5 (red)	
16	31	m	78	178				2.0 (white)	
17	40	m	75	182				2.0 (white)	690
18	23	m	83	186				2.0 (white)	
19	37	m	72	181					670

Results

Ethanol concentration in various beverages

The ethanol concentration in freshly opened commercially available apple juices (by various vendors) varied between 0.1 and 0.4 g/l and did not change significantly, with the exception of one case of one product: its ethanol concentration increased from 0.4 g/l up to 1.1 g/l during 7 days at room temperature (Fig. 1). According to the German regulations for fruit juice, alcohol concentrations up to 3 g/l are allowed. Such high concentrations are merely found in self-made, unpreserved juices.

In freshly opened commercially available grape juices, the ethanol concentration was between 0.3 and 1.8 g/l and increased significantly in two cases during 7 days in storage at room temperature from 0.3 to 0.9 and 0.5 to 1.2 g/l, respectively (Fig. 1).

The ethanol content of the non-alcoholic beers Schöfferhofer Weizen Alkoholfrei and Clausthaler Classic Alkoholfrei was 3.6 g/l in both cases.

In sauerkraut, the ethanol concentration was measured at 2 g/kg. Bananas were stored for 9 days at room temperature and protected from direct sunlight (ESM Fig. 1). On the day of consumption, the matured peeled bananas had an ethanol concentration of 5 g/kg.

EtG in urine after drinking of up to 3 l of non-alcoholic beer

After the consumption of 2.0 to 3.0 l of so-called non-alcoholic beer (c_{EtOH}=3.6 g/l) with an ethanol dose between 7.2 and 10.8 g, the participants' peak concentration of EtG ranged from 0.211 to 0.512 mg/l (EtG_{100} 0.265–0.789 mg/l). The EtS peak concentration ranged from 0.134 to 0.169 mg/l (EtS_{100} 0.217–0.413 mg/l). The concentrations peaked between 5.0 and 7.5 h after drinking, and normalized peak concentrations appeared between 2.5 and 5.0 h after drinking. EtG and EtS were detectable for up to 26 and 25 h, respectively. The concentrations over the time are described in Fig. 2.

An EtG cutoff level of 0.1 mg/l was exceeded in a time period of 3 to 13 h after consumption (1–13 h for EtG_{100}). A cutoff level of 0.5 mg/l was exceeded in a period of 5 to 7 h after drinking (3–7 h for EtG_{100}).

EtG after drinking of up to 2 l of apple juice

After the consumption of 1.1 to 2.0 l of apple juice (c_{EtOH}= 0.3 g/l) with an ethanol dose between 0.3 and 0.6 g, no elevated concentrations were found for EtG or EtS in participants' urine samples (c<LOQ). However, a possibly interfering peak was observed in the LC–MS/MS analysis with a peak concentration approximately 5.5 h after ingestion. Analysis performed under conditions described above was useful in discriminating between a positive EtG result and this interference. The interfering peak was identified by a deviant retention time (t_R (EtG)=4.89 min; t_R (interfering peak)=5.03 min). Additionally, the relative ion ratios were affected (EtG 221/75:221/85:221/113 → 100:100:60; interfering peak 221/75:221/85:221/113 → 100:40:25). The apple juice itself tested negative for EtG/EtS.

EtG after drinking of up to 2 l of grape juice

After the consumption of 1.5 to 2.0 l of white or red grape juice (c_{EtOH}=1.25 g/l (white) and c_{EtOH}=0.6 g/l (red)) with an ethanol dose between 0.9 and 2.5 g, no elevated urinary concentrations were found for EtG (c<LOD mg/l). However, EtS was tested positive with peak concentrations between 0.107 and 0.648 mg/l. The concentrations peaked between 4.5 and 12.5 h after drinking. EtS was detectable for up to 35 h. The concentrations over time are described in Fig. 3.

An LC–MS/MS analysis of white and red grape juices also revealed positive results with EtS concentrations of between 0.03 and 0.124 mg/l.

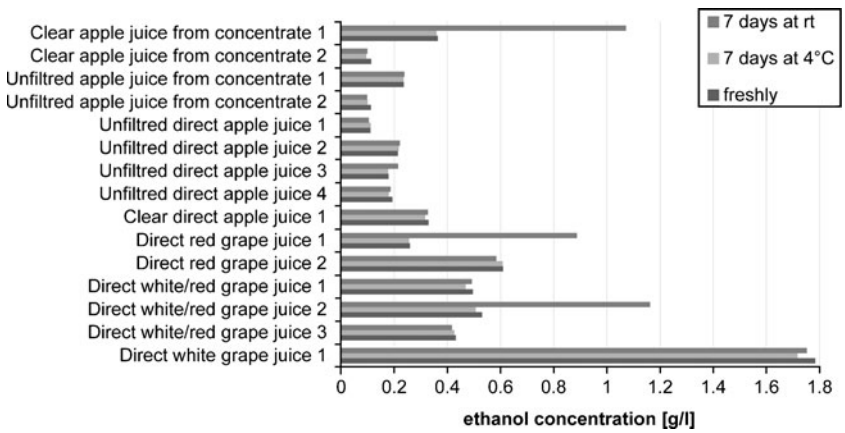

Fig. 1 Ethanol contents of various apple and grape juices freshly opened, 7 days at 4°C and 7 days at room temperature (rt) measured by HS-GC/FID

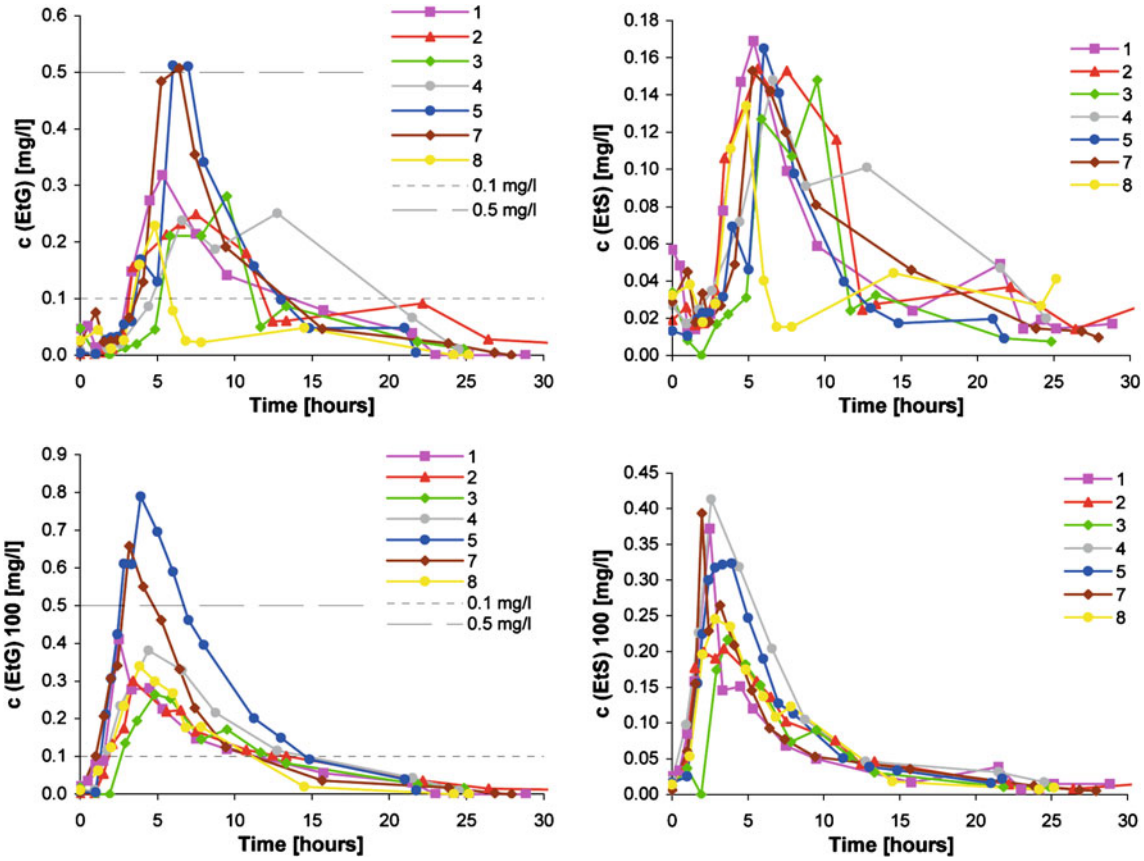

Fig. 2 EtG/EtG$_{100}$ (*left*) and EtS/EtS$_{100}$ (*right*) concentrations in urine over the time after drinking 2–3 l of non-alcoholic beer

EtG after ingestion of sauerkraut

After the ingestion of 750 to 1,320 g of sauerkraut (c_{EtOH}= 2 g/kg) with an ethanol dose between 1.5 and 2.7 g, only in one case was the urinary cutoff level for EtG of 0.1 mg/l reached (Fig. 4). For a participant who ate 750 g, an EtG peak concentration of 0.2 mg/l was measured 2 h after ingestion. The cutoff level was exceeded in a time period of 1.5 to 5.0 h after consumption. EtS concentrations up to 0.055 mg/l were measured in a time period of 2 to 5 h after consumption.

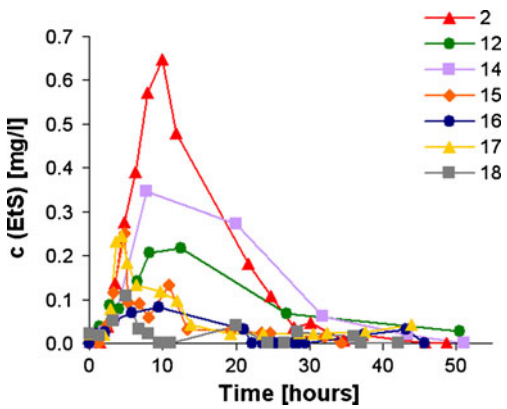

Fig. 3 EtS concentrations in urine over the time after drinking 1.5–2 l of red or white grape juice

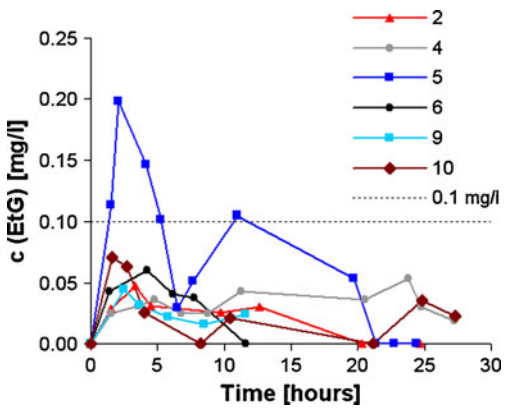

Fig. 4 EtG concentrations in urine over the time after eating 0.75–1.35 kg of sauerkraut

EtG after ingestion of bananas

After the consumption of 670 to 690 g of matured peeled bananas (c_{EtOH}=5 g/kg) with an ethanol dose of 3.5 g, the participants' urinary peak concentration of EtG ranged from 0.04 to 0.12 mg/l. The EtS peak concentration ranged from <0.015 to 0.055 mg/l. The concentrations peaked between 3 and 8 h after ingestion. EtG and EtS were detectable for up to 24 and 20 h, respectively. The concentrations over the time are described in Fig. 5. An EtG cutoff level of 0.1 mg/l was exceeded in two cases in a time period of 3 to 10 h after consumption.

All raw data are summarized in a supplementary file (ESM Table 1).

Discussion

In concordance with the literature and regulations in food law, minor ethanol concentrations were not only found in non-alcoholic beer (according to the German law 0.5 vol% is allowed) but also in fruit juices, sauerkraut, and matured bananas. The alcohol content did not significantly increase in all investigated commercially available fruit juices stored for 7 days at room temperature due to preservatives against further alcoholic fermentation. However, in two products investigated, a slight increase in the ethanol concentration was found, and caution should be taken concerning self-prepared juices that otherwise are not consumable over a longer time period.

In various drinking and eating experiments, the highest alcohol doses were ingested after the consumption of up to 10 g of non-alcoholic beer. After the consumption of fruit juices, sauerkraut, or matured bananas, the alcohol doses ingested were slightly lower. However, positive urinary EtG results were not remarkable and were reported by others after the intake of trace amounts of ethanol. However, in the present study, the peak concentrations and detection windows or time periods with a urinary EtG concentration above 0.1 mg/l were considerable lower.

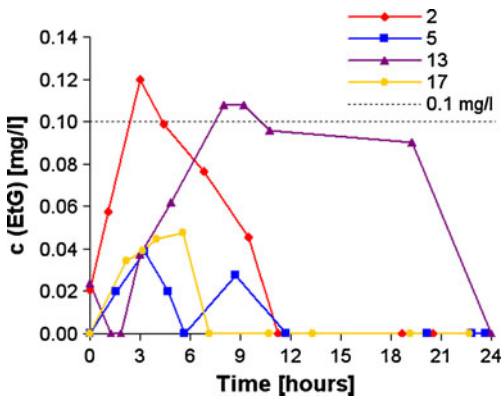

Fig. 5 EtG concentrations in urine over the time after eating 0.8 kg of matured bananas

After the consumption of 9 g ethanol via sparkling wine, the maximum EtG_{100} values ranged from 0.4 to 3.7 mg/l and were reached from 2.7 to 10.0 h [22]. Concentrations above 0.1 mg/l were measured up to 24 h. Recently, after the consumption of trace amounts of alcohol in sparkling wine and whiskey (1 and 3 g), Thierauf et al. [23] found maximum urinary concentrations of 0.49 and 1.36 mg/l for EtG and for EtS normalized to creatinine (EtS_{100}) with 0.15 and 1.17 mg/l, respectively. The concentrations peaked between 2.0 and 6.25 h after drinking. EtG was detectable for up to 9.0 h and EtS for up to 11.3 h. However, in a few cases, an EtG cutoff level of 0.1 mg/l was achieved for up to 5 or 7 h. Consumed alcohol doses and EtG results corresponded well to the present findings after ingestion of fruit juices, bananas, sauerkraut, or so-called non-alcoholic beer.

Also, Høiseth et al. [24] performed drinking experiments using non-alcoholic wine (up to one 0.75-l bottle containing 0.2% ethanol) and low alcohol doses (1.8 g of vodka). After the ingestion of the wine, no positive results were achieved for EtG in the urine samples, but EtS was found positive with up to 2.15 mg/l 5.5 h after ingestion. Surprisingly, EtG and EtS were found in the non-alcoholic wine in concentrations of 3.0 and 1.5 mg/l, respectively. The positive urinary EtS results were attributed to the EtS in the wine, whereas the bioavailability of orally ingested EtG was called into question. After consumption of a similar ethanol dose via vodka, two out of four subjects showed positive urine samples for EtG (c_{max}=0.62 and 0.29 mg/l) and EtS (c_{max} = 0.16 and 0.23 mg/l), peaked 3.5 h after ingestion. Why did the same ethanol dose result in positive findings after the consumption of vodka, while negative results were revealed after the ingestion of non-alcoholic wine? The authors proposed that the ingestion of the wine occurred over a 1-h period, which resulted in lower peak concentrations of ethanol compared with the same dose ingested in one swallow. Additionally, the creatinine concentration has to be taken into consideration.

So-called "innocent" [7] positive urine results for EtG have been evaluated for a group of volunteers who applied Germ-X hand sanitizer (62% ethanol) at varying intervals throughout the day [25]. Only one subject revealed an EtG result above the LOD and reached a urinary concentration of 0.062 mg/l after heavy use of the ethanol-based hand sanitizer every 15 min throughout the workday. This concentration was below a proposed cutoff level of 0.1 mg/l. Considering these results, an alleged EtG urine concentration of 0.75 mg/l after a hand-washing experiment without any information concerning the analytical methods used and all circumstances (e.g., number of washings, times of sampling) is questionable [26]. During a realistic 5-day

exposure period, a hand antiseptic containing 61% ethanol was applied 20 times a day during an 8–12-h waking period with a 20-min minimum time interval between applications [27]. Void urine specimens were collected early each morning for the next 11 days. After starting the experiment, only three out of 97 samples revealed an EtG concentration above 0.1 mg/l (0.106, 0.114, and 0.105 mg/l, respectively).

Costantino et al. [28] performed two studies to evaluate the effect of mouthwash containing ethanol on the appearance of EtG in urine. Nine volunteers were given a 4-oz (120 ml) bottle of a mouthwash containing 12% ethanol. They gargled all 4 oz at intervals over a 15-min period, and urine samples were collected for the next 24 h. Out of 39 specimens, 12 were positive for EtG above 0.1 mg/l, with one sample containing 0.345 mg/l. Peak concentrations were observed within 12 h without normalization to creatinine. In a more realistic second experiment, 11 participants gargled three times a day for 5 days, according to the manufacturer's guidelines. The first morning urinary void was collected every day and two out of 55 samples revealed an EtG concentration above 0.1 mg/l (0.108 and 0.117 mg/l). However, the results were critically discussed concerning the analytical procedures used in the studies but also with respect to well-known pharmacokinetic data [29]. Recently, Høiseth et al. [24] analyzed urine samples for the presence of EtG and EtS of volunteers who rinsed their mouths with 15 ml of a mouthwash solution containing 21.6% ethanol for 1 min and expectorated, followed by a 30-s break. The procedure was repeated eight times, and urine samples were collected 1.5, 3.5, 5.5, and 7.5 h later. All urine samples were negative for EtG and EtS.

The interpretation of urinary EtG results can be critical concerning specificity also for other reasons. On the one site, there is the possibility of a post-collection synthesis of EtG by bacterial contamination (*Escherichia coli*) in the presence of ethanol [30]. A critical collective is diabetics with glucosuria when ethanol can be formed by the fermentation of glucose and then conjugated to EtG by the glucuronidase activity of bacteria, which was also observed in autopsy samples [31]. On the other site, bacterial degradation of EtG was observed and has to be considered [32, 33]. Because of a risk of false identification of alcohol consumption and false-negative EtG results due to bacterial degradation, a simultaneous measurement of EtG combined with EtS, which is not affected by bacterial degradation, was recommended [29, 34]. However, in the present study, EtS was found as a natural ingredient in grape juices, responsible for positive EtS urine findings, and simultaneously EtS as well as EtG were also found in non-alcoholic wine [24]. Other sources cannot be excluded.

In summary, as demonstrated by Rosano and Lin [27], either unrecognized ethanol exposure or endogenous ethanol metabolism can lead to urinary EtG concentrations <0.01 up to 0.08 mg/l. Such a concentration range can also be observed in our own collective of subjects testing negative for EtG in urine using a cutoff level of 0.1 mg/l in the field of driving license regranting. However, these subjects were informed about possible sources of ethanol in foods, medications, or cosmetics/sanitizers prior to the start of the abstinence program and therefore prior to a first urine control (ESM Table 2).

As demonstrated in the present study and discussed in the literature about EtG, concentrations above 0.1 mg/l can be achieved by alternative ethanol sources (foods, cosmetics/sanitizers) but only after an uncommon (ab)use and only for a very limited time period. As demonstrated in various concentrations of the time curves (for non-alcoholic beer), a correction of the urine EtG and EtS concentrations for urine creatinine should be done even after ingestion of large amounts of liquids (formation of EtG_{100} results). Considering a 24-h waiting period between informing a subject and sampling, positive results based on alternative sources for ethanol can normally not be expected; otherwise, a critical examination done by an experienced toxicologist should be helpful in single cases (e.g., job-related or environmental exposure to ethanol or arrival of applicants a few hours after having been informed). Otherwise, it has to be considered that even using a 0.1 mg/l cutoff level and 24-h waiting period is not effective in detecting low-dose alcohol consumption in every case [9]. A higher cutoff level (e.g., 0.3 or 0.5 mg/l) would not be useful in abstinence programs (with a 24-h waiting period) but should be used in other settings with non-informed subjects to avoid not false-positive results—ethanol was present to form EtG—but "innocent positive" findings due to non-drinking exposure to ethanol.

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