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Analysis of 5-hydroxymethylfurfual in coffee, dried fruits and urine

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5-Hydroxymethylfurfural has become a substance of interest since recent results showed a possible carcinogenic potential in consequence of a metabolic activation by sulfotransferases. 5-Hydroxymethylfurfural is formed either by acid catalysed degradation of reducing sugars or via the Maillard reaction. This work provides an overview of foods potentially containing high amounts of 5-hydroxymethylfurfural. It comprises dried fruits with a high sugar content that were exposed to heat for a long time. The concentration ranges from very low in, *e.g.* figs (1 mg/kg) to plums that contained up to 2200 mg/kg. Several types of roasted coffee were analysed that contained from 300 to 2900 mg/kg of 5-hydroxymethylfurfural. In a small human study with seven healthy volunteers the urine excretion of unmetabolised 5-hydroxymethylfurfural was investigated. After uptake of 20 g of plum jam containing 24 mg of 5-hydroxymethylfurfural, 163 µg (mean) were excreted within 6 h, an equivalent of 0.75% of the ingested 5-hydroxymethylfurfural.

Keywords: Dried fruits / 5-Hydroxymethylfurfural / 5-Hydroxymethyl-2-carboxaldehyde / Urinary excretion

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1 Introduction

During processing and storage of carbohydrate containing foods, important chemical modifications can take place. Under acidic conditions occurring in the foods, dehydration of carbohydrates leads to the formation of 5-hydroxymethylfurfural (HMF, 5-hydroxymethyl-2-furancarboxaldehyde). During the dehydration of, *e.g.* fructose to HMF via a sequence of reactions commencing with and retaining the fructofuranose ring intact this compound is formed with and without acid catalysis. A necessary fructofuranosylcation intermediate in this hypothesis is produced directly by the hydrolysis of sucrose, which reacts to produce HMF in high yields. [1].

Moreover, the Maillard reaction can also take place, giving rise to Amadori compounds during the first steps of the reaction, and HMF as a consequence of further reactions. HMF is considered as an indicator compound for the occurrence of 2,3- and 1,2-enolizations in solutions of reducing sugars and is recognised as an indicator of quality deterioration, as a result of excessive heating or storage in a wide range of foods [2, 3, 4] or in sterilised glucose solutions used for parenteral nutrition [5, 6].

The analysis of HMF is normally done by RP chromatography with methanol-water mixtures as eluent with UV detection at 280 nm (*e.g.* [7]). LoCoco and colleagues [8] published a method that implements a precolumns derivatization with 2,4-dinitrophenylhydrazine (DNPH). Derivatization with DNPH gives a better selectivity due to the absorption maximum at 400 nm.

The content of HMF in foods was analysed by several groups, who found very low concentrations in meat and meat products (below 0.9 mg/kg), varying concentrations in bread (up to 410 mg/kg) and coffee (5 to 420 mg/L) and high concentrations in dried fruits and juices from dried fruits (25 to 2900 mg/kg) and in caramel products (110 to 9500 mg/kg). Baby food contained less than 22 mg/kg. Bachmann and co-workers [4] and others [2, 3, 9] estimated based on consumption data that bread and coffee contribute most to the exposure to HMF.

The toxicological relevance of HMF is not elucidated in detail yet. In high concentrations – that are not relevant in nutrition – HMF is cytotoxic and causes irritation to eyes, the upper respiratory tract, the skin and the mucous membrane [10]. Janzowski and co-workers [11] concluded on basis of an estimated uptake of 30 to 60 mg (0.5 – 1 mg/kg

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Abbreviations: DNPH, 2,4-dinitrophenlyhydrazine; **HMF**, 5-hydroxymethylfurfural (5-hydroxymethyl-2-furancarboxaldehyde)**; SMF**, 5-sulfoxymethyl-2-furfural

body weight) that the *in vitro* results do not indicate a health risk. Other publications state that HMF is mutagenic [12] and is transformed by rat and human sulfotransferases to 5-sulfoxymethyl-2-furfural (SMF) which was shown to be mutagenic [13, 14]. In rats, HMF was shown to be completely metabolised to 5-hydroxymethyl-2-furoic acid and N-(5-hydroxymethyl-2-furoyl)glycine. Experiments with radioactive labelled [^{14}C]HMF revealed that most of the radioactivity accumulated in the kidney and the bladder with lower concentrations in the liver [15].

In this work, the analysis of HMF in foods and human urine is presented. Foods containing high amounts of HMF were identified, and a preliminary study with a small group of young healthy adults to investigate the urinary excretion of HMF was carried out.

2 Materials and methods

2.1 Samples and chemicals

The food samples were bought in local markets. All solvents (*e. g*. methanol, ACN) used were of HPLC quality and the other chemicals of analytical grade. They were purchased from Merck (Darmstadt, Germany). Water was distilled twice and further purified using a water purification system (Simplicity, Millipore, Mohlsheim, France). 5-Hydroxymethylfurfural was procured from Fluka (Buchs, Switzerland) and DNPH from Sigma-Aldrich (Vienna, Austria).

2.2 Human study design and subjects

The study group consisted of seven healthy volunteers (age from 18 to 45 years, four male and three female). The evening before the study, the volunteers were asked to avoid HMF-rich foods like coffee, dried fruits, and heated sweet foods. After an overnight fast, the volunteers were given a breakfast consisting of a roll and 20 g of plum jam, which was equivalent of 24 mg of HMF. The urine was collected prior to the breakfast and every hour after the breakfast for 6 h. During the study the volunteers were allowed to drink water but not to eat.

2.3 Sample extraction and cleanup

2.3.1 Dried fruits and fruit bread

Prior to extraction ca. 200 g of the foods were homogenized in a Büchi mixer (B-400, Aargau, Switzerland). From this homogenate, 500 mg was mixed with 1 mL methanol on a Vortex Genie II (Scientific Industries, Bohemia, USA) for 10 min and centrifuged at 13 000 rpm for 10 min. This extract was diluted with water filtered (0.45 μm) and used directly for HPLC analysis. Complete extraction of HMF was tested by consecutive extractions with methanol. As no more HMF could be extracted using a second extraction, only a single extraction was used for the experiments.

2.3.2 Coffee

The extraction of the coffee samples was similar except that water was used as extraction solvent. Since the aqueous extract of coffee contains a lot of polymeric material, an SPE was necessary. The SPE cartridges (Bond elut C18 200 mg, Varian, Harbor City, USA) were conditioned with 5 mL methanol and 3 mL water. Then, 200 μL of the aqueous coffee extract was applied to the SPE cartridges, which were then washed with 3 mL water and eluted with 2 mL aqueous methanol (80%) into a volumetric flask. Prior to analysis, the purified extracts were diluted with water to an appropriate concentration of the analyte.

2.3.3 Urine

Directly after donation the urine samples were put on ice and the sample preparation was started. For analysis of HMF in urine, 3 mL of urine was applied to a 500-mg SPE cartridge. After washing with 3 mL water, the HMF was eluted with 1 mL methanol, which was evaporated with a stream of nitrogen. Then the residue was redissolved in 500 μL water. For derivatization 10 μL DNPH solution (17 mM DNPH in 2 N HCl) was added to the 500 μL and heated to 40°C for 60 min. The samples were cooled immediately on ice and analysed directly after the derivatization.

2.4 Chromatography

The LC was carried out on an HP 1100 (Agilent, Waldbronn, Germany) equipped with a quaternary pump, vacuum degasser, autosampler, and variable wavelength detector. The column was an RP Lichrocart Purosphere Star 100 (55 × 2 mm, 3 μm). The elution was done isocratically with a mixture of methanol (5%) and water (95%) using a flow rate of 0.3 mL/min and an injection volume of 3 μL. The HMF was detected at its absorption maximum at 280 nm.

The derivatized HMF (2,4-dinitrophenylhydrazone) was chromatographed using the same column but eluted with a mixture of methanol (60%) and water (40%) with a detection wavelength of 400 nm. For identification of the derivative in urine LC-MS experiments were carried out using an APCI-MS (Agilent) with the positive ions detected (drying gas temperature 350°C, vaporizer temperature 325°C, drying gas flow 10 L/min, nebulizer pressure 40 psig, capillary voltage 4000 V, capillary current 4 μA). The molecular ion of 305 *m/z* was identified at the same retention time as the

Table 1. Limit of detection and quantification of HMF in water and sample matrix

	Water (ng/mL)	Coffee (ng/g)	Urine (ng/mL)	Dried fruits (ng/g)
LOD	7.0	35	n.d.	3.9
LOQ	23	120	n.d.	13
LOD-DNPH	14	n.d.	2.3	n.d.
LOQ-DNPH	47	n.d.	7.8	n.d.

n.d.: Not determined.

peak occurring using UV detection. For routine analysis, the UV detection was used since we expected a better reproducibility with a lower day-to-day variation.

2.5 Validation and quantification

The validation of the HMF determination was done software supported (Validata, Vers. 3.2) both for the HMF and the DNPH derivative. The limit of detection in water was 7 ng/mL and 14 ng/mL for the HMF (280 nm) and the DNPH derivative (400 nm), respectively (Table 1). The recovery was tested by the standard addition method being 89% for HMF in coffee and only 40% for the DNPH derivative in urine. The reproducibility was checked at a concentration level of 200 ng/mL giving an RSD of 1.4% for HMF and 2.6% for the DNPH derivative ($n = 5$).

Quantification was done by the external standard method using five calibration levels of HMF spanning the expected concentration range. For determination in urine, the HMF standards were derivatized using the same conditions as for urine.

3 Results

The concentration of HMF in the analysed foods was so high that it was necessary to dilute the first extract. The advantage was that no additional clean-up had to be carried out since only one peak occurred in the chromatogram at the conditions used. However, the matrix of roasted coffee was much more complex so that an SPE using C18 cartridges for purification of the extracts was inevitable. As can be seen in Fig. 1 the clean-up resulted in satisfying results.

To obtain an overview of the HMF content in foods some products were analysed that were thought to contain high amounts. The analysed foods comprised products that were heated to high temperatures or for a long time and having a high concentration of reducing sugars. Especially during drying of fruits all these factors apply. Additionally, the traditional preparation of plum jam in Austria is a time-consuming procedure where the fruits are boiled for many hours to gelatinise the fruit pulp and evaporate part of the water. Coffee is normally roasted for only a few minutes but at temperatures up to 300°C. The high acid and sucrose content are the important factors of HMF formation during coffee roasting. The analysis of HMF in foods was necessary because these foods had to be avoided before and during the excretion study.

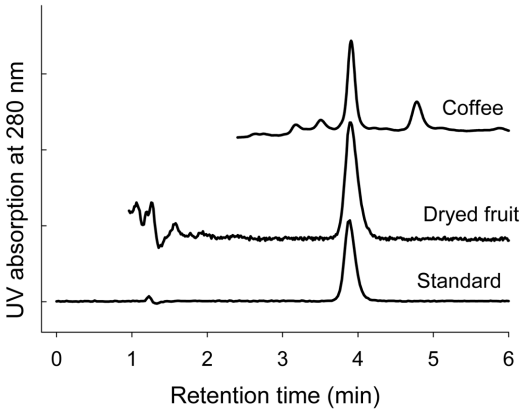

Figure 1. Chromatogram of HMF from dried fruits and roasted coffee.

Table 2. Content of HMF in dried fruits and coffee

Food	HMF content (mg/kg)	No. of samples
Apricot	30; 780	2
Pear	100	1
Peach	40	1
Plum	1600; 2200	2
Plum jam	1100; 1200	2
Date	1000	1
Fig	1	1
Pineapple	280	1
Apple	80	1
Bread with dried fruits	450	1
Coffee (ground, roasted)	300 – 1900	22

The results of the food analysis are collated in Table 2. It can be seen that within the group of dried fruits only a few contain high amounts of HMF. The highest concentrations were found in dried plums (up to 2200 mg/kg) and in a jam made from plums (up to 1200 mg/kg). This jam is produced by very long heating to evaporate part of the water for obtaining the typical taste and paste-like consistency. Dried dates also contained a high concentration of HMF (1000 mg/kg). The two samples of apricots analysed showed quite different content of HMF, namely 30 and 780 mg/kg. This reflects not only a different composition of the products but also a varying drying process. All other dried fruits had significantly less HMF, which could be

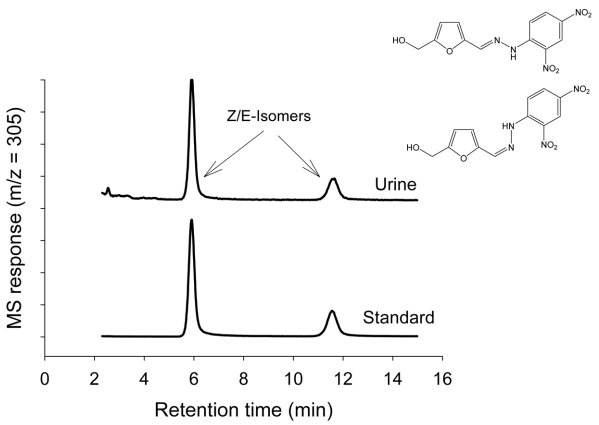

Figure 2. Chromatogram of derivatized HMF from urine using MS detection (SIM: *m/z* = 305).

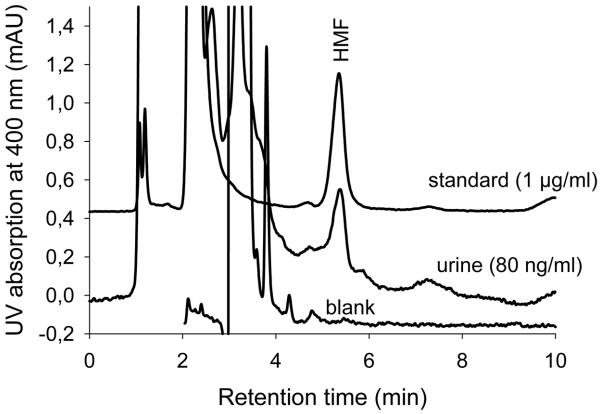

Figure 3. Chromatogram of derivatized HMF from urine using UV detection.

attributed to the different composition or different processing parameters.

The analysis of HMF in urine was developed to estimate the uptake and excretion of this compound. The direct analysis would have been possible with the low limit of detection (7.0 ng/mL) but the high number and concentration of other polar substances in the urine did not give any utilisable peak. For this reason the HMF was derivatized with DNPH that allowed the analysis at 400 nm. The limit of detection of this derivative was higher (14 ng/mL) compared to HMF. Despite this higher LOD, this was sufficient to analyse the excreted unmetabolised HMF.

As can be seen in Fig. 2 the derivatization of HMF resulted in two isomeric forms of the hydrazone that were separated by HPLC. The correct assignment of the (Z,E)-isomers was considered not to be relevant for the analysis. It was shown that at reproducible derivatization conditions (*i. e.* mainly the reaction temperature) the ratio of the two isomers was constant, being 1/0.19. For quantification, only the first peak eluting after ca. 6 min was used not only because of the higher concentration of this isomer but also because no other substance co-eluted at this retention time (Fig. 3). As not all of the HMF is derivatized to a single compound this results in a higher LOD. For routine analysis of HMF in urine, the UV detection was used preferably due to the comparably unstable response of the MS signal.

Table 3. Total amount of HMF (µg) excreted in urine tested every hour after uptake of 20 mg (SD: $n = 7$)

Analysis time (h)	Mean (µg)	Minimum (µg)	Maximum (µg)	SD (µg)
0	0.0	0.0	0.0	0.0
1	7.0	0.0	40	15
2	18	2.0	65	22
3	24	6.0	63	23
4	26	4.0	55	21
5	76	4.0	470	170
6	25	3.0	62	21

4 Discussion

The total uptake of HMF in this experiment was 24 mg originating from the plum jam. Plum jam was used as a food matrix for these experiments because of its homogenous nature and the ability to determine the exact amount of HMF given to the volunteers. The HMF content of the roll contributed with 88 µg (2.4 µg/g) to the total uptake. This low amount of HMF in the roll was neglected during further calculations. The urinary excretion did not show a high variation in most of the subjects. The SD of the excreted HMF of seven subjects can be seen in Table 3. After 5 h one of the subjects had a ca. ten times higher concentration of HMF in the urine that resulted in a significant higher variation and mean excreted amount at this point in time. During the first 6 h that were investigated 163 µg (mean) of unmetabolised HMF – calculated as area under curve (from Table 3) – were found in the urine, which is equivalent to 0.75% of the ingested HMF. The main described metabolites of HMF namely 5-hydroxymethyl-furan-2-carboxylic acid or N-(5-hydroxymethyl-2-furoyl)glycine were not analysed [16].

SMF, the sulfatated metabolite of HMF, was not found in urine. It was analysed by direct injection into the ESI-MS and after HPLC separation. Using the ESI with the negative ions detected the reference substance gave a signal at *m/z* = 205 and 109. Besides not being formed during the human metabolism, one reason could be that SMF is a short-lived metabolite that can react with proteins and DNA or water and does not appear in the urine. Earlier investigations in mice also showed that SMF was not excreted within 48 h.

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