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Occurrence of benzene as heat-induced contaminant of carrot juice for babies in a general survey of beverages

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Abstract

A survey of benzene contamination of 451 beverage samples using headspace sampling in combination with gas chromatography and mass spectrometry (HS-GC/MS) with a quantification limit of 0.13 µg l\(^{-1}\) was conducted. Artefactual benzene formation during headspace sampling was excluded by only mildly heating at 50°C and adjustment of sample pH to 10. The incidence of benzene contamination in soft drinks, beverages for babies, alcopops and beer-mixed drinks was relatively low with average concentrations below the EU drinking water limit of 1 µg l\(^{-1}\). Significantly higher concentrations were only found in carrot juices, with the highest levels in carrot juices specifically intended for infants. About 94% of 33 carrot juices for infants had detectable benzene levels with an average concentration of 1.86 ± 1.05 µg l\(^{-1}\). The benzene contamination of beverages was significantly correlated to iron and copper concentrations, which act as catalyst in benzene formation. The formation of benzene in carrot juices was predominantly caused by a heat-induced mechanism, which explains the higher occurrence in infant carrot juices that are considerably heated to exclude microbiological contamination.

Keywords: Benzene, benzoic acid, beverages, soft drinks, fruit juice, carrot juice, baby food
Introduction

Benzene is one of the contaminants with the highest level of evidence for carcinogenicity. For example, it was classified as carcinogenic to humans (Group 1) by the International Agency for Research on Cancer (IARC 1987). Active as well as passive smoking, automobile exhaust, and driving or riding in automobiles are postulated as the most important pathways for benzene exposure (Wallace 1996). Since high levels of benzene metabolites are frequently reported among children and non-smoking workers without occupational exposure, Johnson et al. (2007) hypothesize that there may significant sources of benzene that are hitherto unidentified.

Since early 1990s, concerns about benzene contamination of food have been raised. Several sources can contribute to the occurrence of benzene in foods. Benzoic acid, a widely used food preservative, may decarboxylate to benzene in the presence of ascorbic acid (Gardner and Lawrence 1993). The formation of benzene from benzoic acid is influenced by the presence of transition-metal catalysts (for example, Cu(II) or Fe(III) ions) and is dependent on pH, UV light or temperature (Gardner and Lawrence 1993; McNeal et al. 1993; Chang and Ku 1993; Barshick et al. 1995). Benzene may be introduced into foods through leaching from various packaging materials or storage environments, from contamination of water supplies, or may be formed during irradiation processes (Barshick et al. 1995). Another source of benzene in soft drinks and beer is contaminated carbon dioxide used for carbonation (Long 1999; Wu et al. 2006).
Methods for determination of benzene in food usually apply GC/MS and different sample clean-up techniques like liquid-liquid extraction (Carrillo-Carrión et al. 2007), static or purge and trap headspace sampling (Page et al. 1992; Gardner and Lawrence 1993; McNeal et al. 1993; Chang and Ku 1993; Barshick et al. 1995; Fabietti et al. 2001; Cao et al. 2007) or headspace solid-phase dynamic extraction (Ridgway et al. 2007). Headspace solid-phase microextraction (HS-SPME) in combination with HPLC was proposed for determination of benzene in fruit juices (Clasadonte et al. 1997). The recent finding of benzene in drinks has raised concerns over analytical methodology, especially heating during the purge-and-trap step that might lead to artefactual formation of benzene (Hileman 2006).

There are only a few surveys that are currently available about benzene in food. Page et al. (1992) determined benzene levels ranging from 0.018 to 3.83 µg kg\(^{-1}\) in 97 Canadian samples of various fruits, juices and drinks. McNeal et al. (1993) showed that food without added benzoates contained lower levels than some foods and beverages containing both ascorbic acid and sodium benzoate. In a study of 60 soft drinks from Italy (Fabietti et al. 2001), none of the samples were found to have benzene levels above the World Health Organization (WHO) guideline value of 10 µg l\(^{-1}\) for benzene in drinking water (WHO 2006). Out of 150 soft drinks sampled in the United Kingdom, 107 did not contain detectable levels of benzene but four contained average levels above 10 µg kg\(^{-1}\) (Food Standards Agency 2006). In a sample of 68 Australian flavoured beverages, 38 contained trace levels of benzene ranging from 1 to 40 µg kg\(^{-1}\) (Anon 2006). From 84 Chinese lager beers, six contained detectable benzene concentrations up to 7.1 µg l\(^{-1}\) (Wu et al. 2006). The U.S. Food and Drug Administration (FDA) tested...
more than 100 soft drinks and other beverages from the United States. Almost all of
which contained either no benzene or levels below 5 µg kg\(^{-1}\) (Meadows 2006). Cao et al.
(2007) assessed benzene levels in 124 Canadian beverages, and found that 40% of the
samples contained detectable benzene levels and two products had benzene levels above
10 µg l\(^{-1}\).

In our study, which is the first survey of benzene in German food products, we
concentrated on product groups that were previously described as likely to contain
benzene such as beverages containing ascorbic and benzoic acid. We focused on
products sold as baby or infant food and intended for the most susceptible consumer
group (Suk et al. 2003). In addition, we included certain alcoholic beverages in the
survey, because ethanol in alcoholic beverages was recently evaluated by the IARC as
carcinogenic to humans (Group 1) (Baan et al. 2007; IARC 2007). The IARC evaluation
emphasizes the importance and necessity of avoiding and controlling carcinogenic
contaminants in alcoholic beverages, since synergistic effects between ethanol and other
contaminants are possible (Lachenmeier 2007). In the case of benzene, such an
interaction with ethanol is likely. This is because significant deviations of acute toxicity
were demonstrated for the binary mixture (Tichý et al. 2002; Rucki and Tichý 2004) and
both substances may be metabolized by cytochrome P450 in humans (Rana and Verma
2005; Seitz and Stickel 2007).
Materials and Methods

Sample collective

Between 2006 and 2007, 451 samples submitted to the CVUA Karlsruhe have been analysed for benzene. As part of official food control in the German federal state Baden-Württemberg, our institute covers the district of Karlsruhe in North Baden (Germany) with a population of approximately 2.7 million. The sampling has been done by local authorities directly at food producers or retail trade. The samples have been randomly selected and collected by government food inspectors, as our institute has requested only the food group and sample number to collect but not specific brands. We had initially requested for food groups “soft drinks”, “alcopops”, “beer-mixed drinks”, and “beverages for babies/infants” that have been previously described to be susceptible to benzene contamination. Due to our risk-oriented sampling approach (Roth et al. 2007), we then increased the sample numbers of conspicuous food groups. For example, after the first detection of comparably high benzene levels in carrot juices, we had specifically requested the food inspectors to collect further samples of carrot juices intended for normal consumption as well as carrot juices intended as baby food for infants and young children in the sense of Commission Directive 2006/125/EC (Commission of the European Communities, 2006).

Quantitative determination of benzene

Benzene was purchased from Sigma-Aldrich (Taufkirchen, Germany). Benzene-d₆ was obtained from Merck (Darmstadt, Germany). The analysis of benzene was done using static headspace (HS) sampling in combination with gas chromatography and mass
spectrometry (GC/MS). For sample preparation, 10 ml of sample was placed in a 20 ml
headspace vial. After adjustment with KOH solution (10%, m/m) to pH 10, the solution
was spiked with 100 µl of benzene-d₆ (100 µg l⁻¹ in methanol) as internal standard. The
vials were tightly sealed and homogenized using a vortex mixer.

The HS-GC/MS system used for analysis was an Agilent model 6890 Series Plus gas
chromatograph in combination with a CTC Combi PAL autosampler and an Agilent
5973N mass selective detector. Acquisition and analysis of data were performed using
standard software supplied by the manufacturer. The samples were incubated in the
agitator oven of the autosampler at 50°C for 30 min. After that, 1000 µL of sample
headspace were injected into the GC/MS system using split injection mode (split ratio
2:1). Substances were separated on a fused silica capillary column (Optima 624, 60 m x
0.25 mm I.D., film thickness 1.4 µm). Helium with a constant flow rate of 1.0 ml min⁻¹
was used as carrier gas. Temperature program: 35°C hold for 1 min, 10°C min⁻¹ up to
240°C, hold for 10 min. The temperatures for the injection port, ion source, quadrupole
and interface were set at 250°C, 230°C, 150°C and 250°C, respectively.

To determine retention times and characteristic mass fragments, electron impact (EI)
mass spectra at 70 eV of the analytes were recorded by total ion monitoring. The
retention time was 11.70 min benzene and 11.65 min for benzene-d₆. For quantitative
analysis, the chosen diagnostic mass fragments were monitored in the selected ion
monitoring (SIM) mode. Benzene: m/z 78 as target ion and m/z 77 as qualifier ion;
benzene-d₆: m/z 84 as target ion and m/z 82 as qualifier ion. For quantification, peak
area ratios of the analytes to the internal standard were calculated as a function of the
concentration of the substances.
The method was validated using two authentic samples analyzed seven times. The precision of the method never exceeded 6.6% (coefficient of variation) and the trueness never exceeded 5.3% (as compared to spiked concentrations), indicating good assay accuracy. Determined according to DIN 32645, the limit of detection was 0.04 µg l\(^{-1}\) and the limit of quantitation was 0.13 µg l\(^{-1}\).

**Quantitative determination of possible benzene precursors and catalysts**

A subset of samples (n=165) was analyzed for benzoic acid, ascorbic acid, copper and iron, which were previously discussed to influence benzene formation in food. Benzoic acid was determined using HPLC according to the German reference method (Anon 1984). L-Ascorbic acid was analyzed using a commercially available enzymatic test-kit (R-Biopharm, Darmstadt, Germany). Copper and iron were analyzed using atomic absorption spectroscopy, according to the German reference method (Anon 1993).

**Experiment about artefactual benzene formation during headspace sampling**

The possible formation of benzene during the headspace sampling procedure was studied using a full-factorial experimental design. The experiment was conducted using a model solution containing sucrose (60 g l\(^{-1}\)), citric acid (4 g l\(^{-1}\)) and ascorbic acid (0.4 g l\(^{-1}\)). Three variables were studied: benzoic acid concentrations (6 levels: 1, 10, 50, 100, 500 and 1000 mg l\(^{-1}\)), pH (2 levels: 3 and 10) and temperature (2 levels: 50°C and 80°C).

**Experiment about heat-induced formation of benzene in carrot juice**
In order to evaluate possible formation mechanisms of benzene in carrot juice with a minimum amount of experiments, the information gleaned from each experiment and the relationships between the experiments have to be fully exploited. This was done using a D-optimal design type (Box et al. 2005; Montgomery 2005). The D-optimal algorithm was used because it chooses an ideal subset of all possible combinations and significantly reduces the number of required experiments compared to standard design types.

The carrot juice used in this experiment was freshly prepared from organic carrots using a juice extractor (centrifuge-type, Starmix, Reichenbach, Germany). The heating temperature was studied at four levels (unheated, 100°C, 125°C, 150°C) and the heating time was varied at three levels (30 min, 60 min, 120 min). The experiments were conducted with 10 ml of juice directly in the headspace vials that were measured afterwards without opening to avoid any loss of benzene.

**Statistics**

The experimental designs and calculations were done using the Software Package Design Expert V6 (Stat-Ease Inc., Minneapolis, Minnesota, USA). The experiments were evaluated using Analysis of Variance (ANOVA) to find the significance of variables and their interactions in the models. The models were checked for consistency by looking at the lack of fit and possible outliers.

The survey data were evaluated using standard statistical packages for Windows. Statistical significance was assumed at below the 0.05 probability level. Groups of two cases were compared using t-tests. One-way ANOVA was used to test whether three or more cases had the same mean including the Bonferroni post hoc means comparison.
Pearson’s test was used to evaluate the significance of linear relations. Box and whisker plots were used for visualization of data (box 25th - 75th percentile, line in the box: median, whiskers: minimum and maximum (max. 1.5 times the length of the inner quartiles), data points outside are outliers).

Results and Discussion

Artefactual benzene formation during headspace sampling

The results of the headspace sampling experiment are shown in Figure 1. The ANOVA results prove that benzoic acid concentration (p=0.0008), as well as pH (p<0.0001) and temperature (p<0.0001) all have a significant influence on benzene formation. However, benzene formation that would lead to false-positive results (that is, results above LOQ of 0.13 µg l⁻¹) only occurs at low pH values in combination with high temperatures and high concentrations of benzoic acid. Our results verify the observation by Cao et al. (2007) that formation of artefacts may occur at 100°C in a 0.5 h time-period, but not at 22-24°C. We suppressed artefactual benzene formation by setting the incubation temperature during headspace GC to 50°C and by pH adjustment of the samples to pH 10. All samples in our market survey and formation experiments were measured under these conditions.

Survey of benzene in German foods

The results of the determination of benzene in different soft drinks, drinks intended for babies and infants (drinks with fruits, vegetables and/or tea), alcopops, beer-mixed
beverages, as well as carrot juices for the general consumer and infants are shown in Table I and Figure 2.

Using ANOVA, it was proved that benzene concentrations in different food groups showed highly significant differences (p<0.0008). The concentrations in carrot juices and especially in carrot juices intended for infants were higher than those in all other groups of beverages. The carrot juices for normal consumption contained average benzene in concentrations below 1 µg l\(^{-1}\), whereas carrot juices intended for infants had significantly (p<0.0001) higher concentrations clearly above 1 µg l\(^{-1}\). Approximately 88% of all carrot juices for infants had benzene contaminations above the EU drinking water limit of 1 µg l\(^{-1}\) (European Council 1998).

The lowest concentrations were detected in other drinks for babies and infants as well as beer-mixed drinks. Soft drinks and alcopops contained slightly higher values. However, only 2% of all soft drink samples had benzene contaminations above the EU limit, and only one sample had a very high benzene concentration of 41.8 µg l\(^{-1}\), which was also above the WHO limit of 10 µg l\(^{-1}\). We were unable to provide an explanation for this high level in the soft drink, and the levels of the analyzed precursors and catalysts were inconspicuous. The samples from the same product charge, which were simultaneously analyzed, had benzene levels below 1 µg l\(^{-1}\).
Precursors and catalysts influencing benzene formation in beverages

A multivariate data analysis was conducted to gain a first overview of the dataset of 165 samples analyzed for benzene and the possible precursors and catalysts benzoic acid, ascorbic acid, copper and iron. The results of the principal components analysis (PCA) are shown in Figure 3. The correlation loadings plot shows that benzene appears to be closely correlated to copper and iron, which influence the PC1 component. Benzoic acid and ascorbic acid are inversely correlated on the PC2 component. The results of PCA were confirmed by univariate data analysis (Table II). The copper and iron concentrations were significantly higher in benzene-positive samples than in benzene-negative samples. Table III shows that the concentration of benzene has a relation to that of copper and iron. However, there also appear to be other - so far unknown - influences as the metal concentrations show a large dispersion, and some benzene-free cases had higher metal concentrations than benzene-positive ones. The influence of copper and iron was not unexpected due to the experiments in model solutions by Gardner et al. (1993). Our study suggests that a metal-catalyzed formation mechanism at least influences benzene formation in real food. We also can partially confirm the observation of Gardner et al. (1993) that there may possibly be non-linear effects or confounding between different constituents on the formation on benzene. For example, Gardner et al. (1993) detected a maximum of benzene production at 1.0 nM CuSO₄, higher concentrations of this metal resulted in a small decrease in benzene concentration. And even when there was no CuSO₄ in the solution, significant amounts of benzene were still produced. In the same study, it was also observed that addition of FeSO₄ in the absence of CuSO₄ led to benzene formation, but at higher concentrations of FeSO₄ the benzene production decreased. In model solutions, there appears to be a dependency
between the concentrations of the various constituents and benzene formation. The same appears to be true for our real food matrices; however, the analysis of our data was unable to detect interactions between both metals or a certain metal concentration that leads to maximum benzene formation. Presumably, other influences (e.g. temperature) may have larger influences than the metals and lead to confounding of the data.

[Insert Figure 3 & Tables II and III about here]

In contrast to the clear influence of copper and iron, benzoic acid concentration tended to be higher in benzene positive samples but not on a statistically significant level (Table III). The PCA scores plot (Figure 3) shows that only some soft drink samples containing benzoic acid were also high in benzene. But our results show that the presence of benzoic acid was not causative for benzene formation in our samples and that other mechanisms appeared to be more predominant. No relation at all could be proven between ascorbic acid and benzene. Our results proved the inconsistency detected between levels of benzene and benzoate salts or ascorbic acid previously described by Cao et al. (2007). In contrast, earlier studies had confirmed a clear connection between benzoic acid and benzene, which was dependent on the concentration of benzoic acid in the beverages and showed large interactions with the concentration of ascorbic acid (Gardner et al., 1993; Page et al. 1992; McNeal et al., 1993). Therefore, it is likely that due to the efforts of the beverage industry to avoid benzoic acid especially in combination with ascorbic acid, the benzene formation mechanism from those precursors is nowadays only of secondary relevance, but still may be causative if high concentrations of benzoic acid are used as preservative.
Metal catalyzed and heat-induced formation of benzene in carrot juices

None of the carrot juices in our study contained benzoic acid. However, both groups of carrot juices contained higher concentrations of the investigated metals than the soft drinks (p<0.0001 for copper and iron). Therefore, a metal catalyzed formation of benzene from so far unknown aromatic precursors may be at least partially causative in the case of carrot juice.

Due to the differences of benzene concentration in carrot juices for general consumption and for infants described above, the question of formation mechanism in this special kind of vegetable juice arose. A metal-catalyzed mechanism alone could not explain the higher concentrations in carrot juices for infants, since both groups of carrot juices had the same levels of metals (p=0.5245 for iron, and p=0.0900 for copper).

Our working theory was that infant juices were heated more extensively, which led to heat-induced formation of benzene similar to the well-characterised heat-induced formation of other food contaminants like acrylamide or furan (Wenzl et al. 2007).

The rationale for the extended heating of carrot juice for infants is to minimize the microbiological risk. A typical sterilisation process of carrot juices for infants involves autoclaving at 122°C for 45 min at 2.2 bar, whereas normal carrot juices are typically heated at 120°C for only 5 min at normal pressure.

The results of our experiments about heat-induced formation of benzene in carrot juice (Figure 4) show that the formation in fact significantly depends on temperature
The formation of benzene in carrot juices appears to be predominantly heat-induced. It is so far unknown if the metals play a role in this heat-induced mechanism. Further research is needed to determine if elimination of the metals could minimize the benzene formation during the sterilisation step.

Food toxicological evaluation

Besides a few exceptions, benzene exposure of the consumer due to soft drinks and alcoholic beverages on the German market appears to be very low and nearly negligible in consideration of the exposition to benzene from other sources. According to Council Regulation (EEC) No 315/93 laying down Community procedures for contaminants in food (Council of the European Communities 1993), no food containing a contaminant in an amount unacceptable from the public health viewpoint and in particular at a toxicological level shall be placed on the market. Furthermore, contaminant levels shall be kept as low as reasonably can be achieved by following good practices.

It is also reasonable to apply the EU drinking water limit of 1 µg l$^{-1}$ as basis to evaluate other beverages. According to our results, it is possible to manufacture beverages with benzene contamination below 0.5 µg l$^{-1}$. Therefore we think that soft drinks with benzene contamination above 1 µg l$^{-1}$ are in offence against Council Regulation (EEC) No 315/93 and should be removed from the market.
Conclusion

In general, our study confirms the established view that food is not an important pathway for benzene exposure (Wallace 1996). However, some food groups seem to have a higher risk of benzene contamination than others, and a systematic study of exposure to benzene in the complete human food chain is missing so far. In our study, carrot juices formed the food group with the highest susceptibility to benzene contamination due to heat-induced formation. In the nutrition of babies and infants, carrot juices belong to the standard program as a complimentary food for infants from four months, when breast milk or infant formulas alone can no longer meet the baby’s growing nutritional requirements. For this reason, significant consumption of carrot juice per kg of bodyweight is possible in infants. It would be certainly interesting to study if the exposure to benzene containing baby food may provide an answer to the elevated levels of benzene biomarkers in children, whose origin was so far unexplained. (Johnson et al. 2007). As toxicology does not allow establishment of safe benzene levels above zero, any exposure at all to benzene and benzene-containing products should be avoided (Mehlman 2002). This is especially true in the case of exposure of children to benzene. Since there are two infant carrot juices without benzene contamination in our survey, it appears to be technologically possible to manufacture such juices without benzene formation. The manufacturers of carrot juice should optimize their processes with priority.

Further research concerning precursors of heat-induced benzene formation is necessary and food surveillance should introduce carrot and other vegetable-containing infant products into market surveys.
References


http://mc.manuscriptcentral.com/tfac Email: fac@tandf.co.uk


Figure Legends

Figure 1. Artefactual benzene formation during headspace analysis. The experiment was conducted using a model solution containing sucrose, citric acid, ascorbic acid and different amounts of benzoic acid at 2 different pH values and 2 different temperatures. Significant artefactual benzene formation was only detected at pH 3, 80°C and benzoic acid concentration above 10 mg l⁻¹.

Figure 2. Box-plots of the benzene concentrations in different product groups. Carrot juices and especially those intended for infants contained significantly higher benzene concentrations than all other product groups.

Figure 3. PCA scores and correlation loadings plot of a dataset of 165 alcohol-free drinks analyzed for benzene, benzoic acid, ascorbic acid, copper and iron. Three categories are marked in the scores plot (soft drinks, carrot juices, infant carrot juices). The correlation loadings show that benzene is highly correlated to both iron and copper concentrations.

Figure 4. Heat-induced formation of benzene in carrot juice. Significant amounts of benzene are formed if the carrot juice is heated at temperatures higher than 100°C for over 30 min. Freshly pressed organic carrots without any additives were used for this experiment.