Journal of Chromatography A, 1211 (2008) 113–119

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Headspace solid-phase microextraction–gas chromatography–mass spectrometry for the quantitative determination of the characteristic flavouring agent eugenol in serum samples after enzymatic cleavage to validate post-offence alcohol drinking claims

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ARTICLE INFO

Article history:
Received 25 July 2008
Received in revised form
18 September 2008
Accepted 22 September 2008
Available online 25 September 2008

Keywords:
Alcoholic beverages
Flavouring agents
Congener analysis
Beverage-characteristic aroma compounds
Eugenol
Bitter
HS-SPME–GC–MS
Metabolism

ABSTRACT

A rapid headspace solid-phase microextraction–gas chromatography–mass spectrometry (HS-SPME–GC–MS) method has been developed for the determination of eugenol in serum samples after enzymatic cleavage. Eugenol is a characteristic marker for the consumption of certain alcoholic beverages including some digestif bitters and herbal liqueurs as well as wood-cask-aged spirits. This method enables the detection of eugenol with a limit of detection (LOD) of 3.2 ng/ml and a limit of quantification (LOQ) of 4.8 ng/ml in serum samples with excellent precision (5.3% intraday, 6.9% interday) and linearity (correlation coefficient $R^2 = 0.992$). Our findings confirm that eugenol undergoes a rapid phase II metabolism as it occurs completely conjugated as eugenol glucuronide in serum. Free eugenol was not detectable in any of our samples, which necessitated enzymatic cleavage with β-glucuronidase prior to HS-SPME sampling. *In vivo* experiments were conducted with a volunteer, who consumed a digestif bitter beverage on three different days under controlled conditions. At defined intervals, blood samples were taken from the subject. Using these blood samples, concentration/time profiles for serum eugenol glucuronide were determined. A rapid resorption leads to a peak eugenol glucuronide concentration directly after drinking (up to 1742 ng/ml if 78 mg of eugenol are ingested) followed by a decrease during the next 3 h. Blood samples were also taken from 20 drivers claiming to have consumed drinks containing eugenol. In five of the samples, eugenol glucuronide was detected at serum concentrations ranging from 12.1 to 172.3 ng/ml. These test results, in particular, confirm that the analysis of volatile compounds can be useful in forensic toxicology for the verification of post-offence alcohol consumption claims.

1. Introduction

In Germany, Article 103 (2) of the Basic Constitutional Law prescribes that if the driver of a motor vehicle is to be charged with a drinking-and-driving offence, the blood alcohol concentration detected after the offence must have been present at the time of the offence itself, and that this concentration must be at a punishable level. If the defendant claims to have drunk the alcohol only after the offence, and to have been sober at the time of the accident, the plausibility of this claim sometimes can be verified by means of the analysis of congener substances and the claim, where applicable, disproved. The term 'congener substances' summarises all other volatile compounds, besides ethanol, contained in alcoholic beverages. A detailed description of congener analysis, introduced by Machata and Prokop [1] and extended by Bonte and Busse [2], is listed in corresponding literature [3–6]. Typically, methanol and higher alcohols have been used as congener substances to validate the consumption of certain alcoholic beverages, but sometimes the specificity of these substances is lacking, because nearly all alcoholic beverages contain these alcohols, albeit with very large

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doi:10.1016/j.chroma.2008.09.068

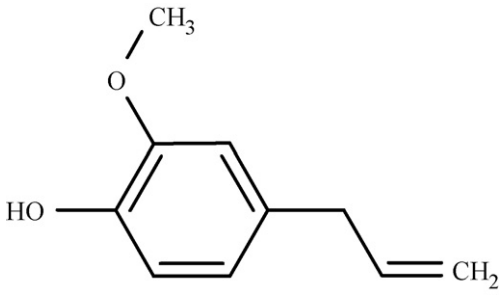

Fig. 1. Chemical structure of eugenol [97-53-0] $C_{10}H_{12}O_2$.

variations in content [7]. Our group was the first to suggest substances other than alcohols for the purpose of congener analysis, focusing on substances that are specific for only a certain category of alcoholic beverages. For example, thujone was evaluated to provide proof of the consumption of absinthe. But we were not able to detect thujone in blood samples because of the very low concentrations in the spirit itself [8]. However, we were recently successful in determining the presence of anethole in serum samples, which is a specific marker for the consumption of aniseed spirits [9]. The current study now proposes eugenol as novel marker unique to certain bitters and herbal liqueurs.

Eugenol (4-allyl-2-methoxyphenol; $C_{10}H_{12}O_2$; CAS 97-53-0) (Fig. 1) is a semivolatile compound found naturally in some herbs. It is the major component (80%) of the essential oils in the buds of clove (*Flores caryophylli*) and in the bay berry (allspice) of the pimento tree (*Fructus pimentae*, 60–90% [10]). Eugenol is also found in basil, cinnamon, fennel, marjoram, nutmeg, anise, and tarragon [11–13]. Digestions and distillates of these plants are used as aromatic substances in alcoholic beverages such as bitters, herbal liqueurs and spice liqueurs [10].

A HPLC method for separation and electrochemical detection of eugenol in spirits has been described in literature [14]. The detection of eugenol in other materials, e.g. in mainstream cigarette smoke [15], is also possible. However, in a previous investigation of spiked blood samples, we were unable to detect eugenol with dynamic headspace GC–MS at the required sensitivity [16]. Therefore, in this study we have introduced a sample enrichment protocol using headspace solid-phase microextraction (SPME), prior to GC–MS, to allow quantitative trace analysis of eugenol in serum samples. Our aim is to be able to prove or disprove alcohol consumption claims in criminal forensic cases.

2. Experimental

2.1. Reagents and standards

Eugenol, Na_2SO_4 and ethanol were purchased from Merck (Darmstadt, Germany) and dicyclohexylmethanol as an internal standard was obtained from Sigma–Aldrich (Steinheim, Germany). β-Glucuronidase for enzymatic cleavage was purchased from Roche Diagnostic, Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade. Water was deionised. Negative control serum samples for spiking with eugenol were taken from the authors.

2.2. Sample preparation

A standard solution of 200 and 2000 ng/ml of eugenol was made in water with ethanol as solubilizer. The aqueous standard solution must be freshly prepared, because it is instable. The serum stock solution of eugenol was prepared by addition of 1 ml eugenol solution (aqueous standard 200, 2000 and 20,000 ng/ml, respectively) to 9 ml of negative control serum sample. Subsequent solutions for calibration curves and validation parameters were accomplished by adding standard stock solution to negative control serum in resulting concentrations of 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20, 50, 100, 200, 1000, 2000 ng/ml (ppb) eugenol. The serum stock solutions were also freshly prepared. For accuracy and precision tests interday, a serum stock solution with 20 ppb eugenol was frozen at −18 °C. During the routine use of the assay, this 20 ppb sample was also applied as quality control sample.

2.3. Headspace-SPME procedure

SPME experiments were performed using a manual fibre holder supplied by Supelco (Taufkirchen, Germany). Four commercially available fibres, Carbowax/divinylbenzene (CW/DVB, 65 μm), Stable-Flex Carboxen/polydimethylsiloxane (CAR/PDMS, 85 μm), Stable-Flex polydimethylsiloxane/divinylbenzene (PDMS/DVB, 65 μm) and Stable-Flex divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) were purchased from Supelco. Before use, each fibre was conditioned in the GC injection port under helium flow in accordance with the temperature and time recommended by the manufacturer. Fibre blanks were run periodically to ensure the absence of contaminants or carryover.

The SPME procedure without enzymatic cleavage was as follows: 200 μl serum and 200 μl internal standard solution (100 ng/ml dicyclohexylmethanol) were placed in a 22 ml headspace-vial containing an 8 mm × 3 mm PTFE-coated stir bar and 0.1 g Na_2SO_4. The samples were immediately sealed with silicone-PTFE septa. Before HS-SPME analysis, the sample vial was stirred for 1 min and conditioned for 1 min in a thermostatic water bath at a temperature of 50 °C. Then the sample was extracted using PDMS/DVB (60 μm) fibre for 30 min at 50 °C and a magnetic agitation rate of 700 rpm. The thermal desorption of the analyte was carried out by exposing the fibre in the GC injection port at 250 °C for 3 min. To prevent a memory effect, the fibre was kept in the injection port for an additional time of 7 min in the split mode (purge on).

For sample preparation with enzymatic cleavage, 20 μl internal standard solution (1000 ng/ml dicyclohexylmethanol), 175 μl buffer (phosphate buffer, 0.1 M, pH = 6.0) and 5 μl β-glucuronidase were used instead of 200 μl internal standard solution. The enzyme was added immediately before the SPME enrichment. The incubation time corresponds to the extraction time of 30 min.

2.4. Beverage analysis

The testing of spirits for eugenol content was conducted using a headspace-trap procedure. The headspace analysis was performed with the Perkin-Elmer TurboMatrix HS 110-trap automatic headspace sampler with trap enrichment and flame ionisation detector (Perkin-Elmer, Shelton, CT, USA). A capillary column Rtx 1701 (60 m × 0.530 mm I.D.; 1.5 μm film thickness) with phenylcyanopropyl phase from Restek was used. Data acquisition and integration were carried out with TotalChrom (Version 6.2.1) software. The enrichment conditions and chromatographic conditions were previously described in detail [17].

2.5. GC–MS conditions

The GC–MS system used for analysis was a Hewlett-Packard GC 5890 series II with a 5971 mass selective detector (Waldbronn, Germany). Data acquisition and analysis were performed using standard software supplied by the manufacturer. Substances were separated on a fused silica capillary column HP-5MS