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Sensitive biomonitoring of monoterpene exposure by gas chromatographic–mass spectrometric measurement of hydroxy terpenes in urine

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Abstract

A gas chromatographic method with mass selective detection was developed which enables the simultaneous determination of the urinary hydroxy terpenes *cis*-verbenol, α-terpineol, myrtenol, carveol, perillyl alcohol and *trans*-sobrerol. The sample preparation consisted of enzymatic hydrolysis, solid phase extraction (SPE) with RP-C_{18} SPE material and clean up with silica gel cartridges. Large volume injection was used and the mass selective detection was done in the single ion modus. Low detection limits in the range of 1.0–4.5 μg/l for the terpene metabolites in urine and mean recoveries of 100% were achieved.

Keywords: Biomonitoring; Monoterpenes; Hydroxyterpenes

1. Introduction

High monoterpene concentrations of α-pinene, β-pinene, Δ^3-carene and limonene in the air may irritate the skin, eyes and mucous membranes and prolonged exposure may result in allergic contact dermatitis or chronic lung function impairment [1–5]. Occupational exposure to α-pinene, β-pinene and Δ^3-carene in the mg/m^3 range occurs at woodworking places for example during sawing of pine wood [6,7]. Airway irritations have been described at such working places [6–8]. Environmental monoterpene exposure in the μg/m^3 range is caused by the release from building material of wood, paints and varnishes, cleaning agents and cosmetics in the indoor environment. Because of these various exposure sources monoterpenes are one of the most frequent group of volatile organic compounds (VOC) in the indoor environment in Germany [9–11]. In contrast to other compounds like aromatic hydrocarbons, in the recent years the indoor air terpene concentrations are increasing, obviously because of the increasing use of natural products and wood furniture.

Biological monitoring of monoterpene metabolites in the biological fluids blood and urine provides a useful method for determination of the individual's uptake of monoterpenes [12]. Thereby bioavailability of the terpenes is also taken into account. Until now biological monitoring of monoterpenes in urine is

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only studied by the renal excretion of verbenols, metabolites of α-pinene [13]. Good correlations were found between exposure to α-pinene and verbenol concentrations in the urine of workers in joinery shops and sawmills [6,7]. However the described method was not suitable for the sensitive determination of low terpene exposure [14]. The aim of this study was to develop a sensitive GC–MS method for the simultaneous determination of several metabolites of the main relevant monoterpenes derived from the indoor air environment. Verbenol and myrtenol were selected as metabolites of α-pinene [15], carveol and perillyl alcohol as metabolites of limonene [16] and *trans*-sobrerol and α-terpineol as possible metabolites of α-pinene or terpinenes.

2. Experimental

2.1. Chemicals

cis-Verbenol, α-terpineol, myrtenol and perillyl alcohol were obtained from Fluka (Buchs, Switzerland), carveol and *trans*-sobrerol from Aldrich (Milwaukee, WI, USA) and β-glucuronidase/arylsulfatase from Merck (Darmstadt, Germany). The acetonitrile (J.T. Baker, Deventer, The Netherlands) and ethyl acetate, methanol, acetone, dichloromethane, pentane, cyclohexane, *n*-heptane and toluene (Merck) used were gradient grade. Water were obtained from a Milli-Q water purification system (Millipore, Eschborn, Germany). RP-C_{18}-SPE material (particle size 40 μm, pore size 600 nm, 18.5% carbon coating, 480 m^2/g active surface area, J.T. Baker) were used for extraction and silica-gel (particle size 0.063–0.200 nm, for column chromatography, Fluka) for purification.

2.2. Sample preparation

The sample preparation was performed according to Ref. [17]. Pool urine samples were stored at −20 °C until sample preparation. Twenty ml defrosted pool urine were added into a 100-ml Erlenmeyer flask with 10 ml 0.1 M sodium acetate buffer (pH 5.0) and 25 μl β-glucuronidase [E.C.3.2.1.31]/arylsulfatase [E.C.3.1.6.1] (30 and 20 Units/ml, respectively). For hydrolysis of the conjugates the mixture was shaken at 120 rpm and 37 °C for 16 h. The hydrolyzed pool urine was applied to a 3-ml SPE glass cartridge (50 mm×8.5 mm I.D.) with a polytetrafluoroethylene (PTFE) frit and 300 mg RP-C_{18}-SPE material. Before use all parts of the SPE device were cleaned by pentane–acetone (2:1, v/v) mixture. The RP-C_{18}-SPE material was conditioned with 2.5 ml methanol followed by 5.0 ml 5% methanol–water solution (v/v). A PTFE tube (25 cm×3 mm I.D.) was fixed on the SPE cartridge by a PTFE cartridge–tube adapter and used for loading the RP-C_{18}-SPE material with the hydrolyzed pool urine sample directly from the incubation flasks by a flow of approximately 10 ml/min generated by water-pump vacuum. After washing twice with 2.5 ml 5% methanol–water solution (v/v) the RP-C_{18}-SPE material was dried under water-pump vacuum and a gentle stream of nitrogen for 1 h. The analytes were eluted from the dry RP-C_{18}-SPE material in steps of approximately 200 μl by ethyl acetate. A total volume of 2.0 ml was collected in volumetric flasks.

For further purification 300 mg silica gel was suspended in ethyl acetate and poured into 3-ml SPE glass cartridges. The 2.0 ml ethyl acetate urine extracts were directly and quantitatively transferred to the silica gel cartridge followed by further approximately 0.5 ml ethyl acetate. Two ml extracts were eluted and collected in 2.0 ml volumetric flasks. The extract received were stored in vials with a PTFE-lined cap at +4 °C until GC–MS-analysis.

The experiments for each condition were performed 3–5-fold and the data received were averaged out.

2.3. GC–MS method

The GC–MS system consisted of a Gerstel programmable temperature vaporiser (PTV)/Gerstel MPS large volume sampler (CIS 3, Gerstel, Mühlheim a.d. Ruhr, Germany), a HP GC 5860 series II (Hewlett-Packard, Waldbronn, Germany), and a HP MSD 5972 (Hewlett-Packard). Forty μl of the urine extracts were injected with an injection speed of 29 μl/min into the liner of the PTV containing silanized glass wool (93 mm×1 mm I.D., Gerstel) in the solvent vent mode with stop flow (vent flow, 200 ml/min helium). After purging of the

organic solvent over 0.5 min at 20 °C and further 0.5 min isotherm the injector was heated at 600 °C/min to 300 °C and was kept 2 min isotherm in the splitless mode. The GC–MS transfer line was held at 300 °C resulting in a quadrupole temperature of 180 °C. The separation was achieved with a 30.0-m×280-μm I.D. Rtx®-5Sil MS GC-column (0.25 μm 5% dimethyl–95% diphenylpolysiloxane, Restek, Bellefonte, PA, USA) and helium was used as carrier gas. The gas chromatographic separation was performed by a temperature gradient; 1.0 min isotherm at 37 °C, 15.0 °C/min up to 290 °C, 20.0 °C/min up to 330 °C and 1.0 min isotherm at 0.7 bar column head pressure. The single ion modus (SIM) was used to detect the different terpene metabolites. A time program monitors usually two specific masses of the analytes in different time windows for optimal, sensitive and selective detection. The SIM time program started at 6.00–8.00 min, *m/z* 94.0, 109.0; 8.00–9.00 min, *m/z* 84.0, 93.0, 109.0; 9.00–9.85 min, *m/z* 79.0, 93.0 and 9.85 min up to the end, *m/z* 79.0, 109.0.

3. Results and discussion

3.1. Sample preparation

The removal of urine matrix compounds as well as the enrichment of the analyte concentration is necessary for clear identification and sensitive quantification of hydroxy terpenes. Different washing solvents for the RP-C_{18}-SPE material were tested for their efficiency to eliminate matrix compounds without affecting the recovery. First different methanol–water mixtures were investigated as washing solvents but more than 10% methanol (v/v) elutes parts of *cis*-verbenol and *trans*-sobrerol from the RP-C_{18}-SPE material. Washing by alkaline (2% ammonia hydroxide in 5% methanol–water) and acid solutions (2% acetic acid in 5% methanol–water) followed by neutral washing with 5% methanol–water, respectively, decreased the recovery of all terpene metabolites especially *cis*-verbenol. Therefore, further washing procedures were carried out with 5% methanol–water solution.

Several solvents with different properties and polarities were used for the elution of the terpene metabolites from the RP-C_{18}-SPE material (Table 1). Recoveries were gained by comparison of standard solutions prepared in methanol with the results of spiked urine samples. Elution with ethyl acetate and acetonitrile showed the best recovery rates. Non-polar organic solvents like cyclohexane, *n*-heptane, and toluene only shows recovery rates of 10–20%. However, heptane were used for the elution of verbenol described elsewhere [14]. Acetone extracts were coloured dark yellow indicating elution of urine matrix compounds. Some matrix compounds co-eluted with the terpene metabolites especially with *trans*-sobrerol during the GC separation leading to an over estimation of the analyte concentrations.

In order to avoid interference of the GC separation by matrix compounds, the ethyl acetate extracts received were further purified by clean up with the polar adsorbent silica gel. Compared to the sample preparation without silica gel clean up the contamination of the liner (injection port) with non-volatile urine matrix compounds was reduced and memory effects were not observed. The background of GC–

Table 1
Influence of the solvent used for elution from the SPE cartridge on the recovery rates of the hydroxy terpenes (250 μg/l urine, sample preparation without silica gel clean up)

	Recoveries (%)					
	cis-Verbenol	α-Terpineol	Myrtenol	Carveol	Perillyl alcohol	*trans*-Sobrerol
Ethyl acetate	71±7	113±5	107±3	110±2	107±2	143±6
Acetonitrile	65±19	96±10	98±12	96±9	97±2	135±6
Acetone	116±23	150±4	144±4	143±5	114±2	131±10
Dichloromethane	29±15	37±20	51±15	52±15	74±4	16±4
Cyclohexane	13±10	15±7	14±15	14±16	10±8	6±12
n-Heptane	14±9	16±14	17±15	18±14	13±15	7±10
Toluene	13±11	15±13	13±6	14±12	10±9	6±8

MS chromatograms was reduced avoiding coelution of matrix compounds with hydroxy terpenes. This gave also for spiked urine samples with lower hydroxy terpene concentrations mean recoveries of 100% (Table 2).

Since further analyte enrichment by evaporation of the ethyl acetate using a gentle nitrogen stream resulted in analyte loss, it was investigated which solvent volume is sufficient for complete analyte elution from the SPE cartridge. Fig. 1 shows the fractional elution in 200-μl steps of the analytes from the SPE cartridge by the use of ethyl acetate. The collected volumes were calculated by weight and density of ethyl acetate. Two ml ethyl acetate are sufficient for the complete extraction of all hydroxy terpenes. This corresponds to an enrichment factor of 10 starting from 20 ml urine.

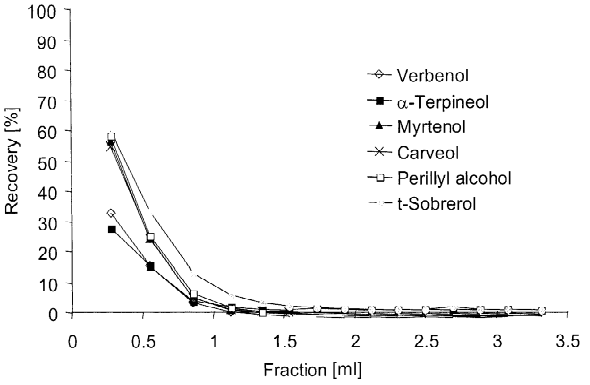

Fig. 1. Fractional elution of the hydroxy terpenes from the SPE cartridge by ethyl acetate.

3.2. GC–MS-analysis

Fig. 2 shows the GC–MS-chromatograms of a pool urine spiked with terpene metabolites prepared under the conditions described above (Fig. 2B) as well as an external standard mixture (Fig. 2A). The complete gas chromatographic separation of the six hydroxy terpenes takes place within 10 min (Table 3). The selectivity of the gas chromatographic separation is high enough for baseline separation of the analyte signals. The identification of the analytes was based on the retention time and the selective mass-fragments determined by the SIM time program (Table 3). The sensitivity of the GC–MS determination was improved by increasing the injection volume. Large volume injection using a PTV injector is based on the injection of extracts and selective evaporation of the solvent from the liner while simultaneously the less-volatile compounds are trapped in the cold liner [18,19]. An increase of the injection volume up to 40 μl urine extract was possible without significant contamination of the GC–MS system by matrix compounds.

3.3. Validation of the method

To demonstrate the reliability of the developed method, recoveries, correlation coefficients, detection and quantitation limits were determined using spiked urine samples as described in the experimental section. The recoveries of hydroxy terpenes (each 25 μg/l urine) with silica gel clean up using ethyl acetate as extraction solvent are in the range of 93–118%, except for *trans*-sobrerol. The higher polarity of *trans*-sobrerol caused by the two hy-

Table 2
Recoveries, detection and quantitation limits of the hydroxy terpenes in urine

	Recoveries[a] (25 μg/l urine) (%)	Detection limit (DIN 32 645) (μg/l)	Quantitation limit (DIN 32 645) (μg/l)	Detection limit ($S/N>3$) (μg/l)	Standard deviation of the method (μg/l)	Correlation coefficient
cis-Verbenol	107±10	1.9	6.6	2.1	0.6	0.987
α-Terpineol	108±4	2.5	14.0	2.0	0.9	0.984
Myrtenol	98±5	1.3	4.4	2.0	0.3	0.984
Carveol	93±6	1.9	7.0	1.0	0.6	0.980
Perillyl alcohol	118±7	4.5	21.8	1.1	1.7	0.983
trans-Sobrerol	85±11	2.5	9.0	1.5	0.7	0.987

[a] Elution of the SPE cartridge with ethyl acetate, silica gel clean up.

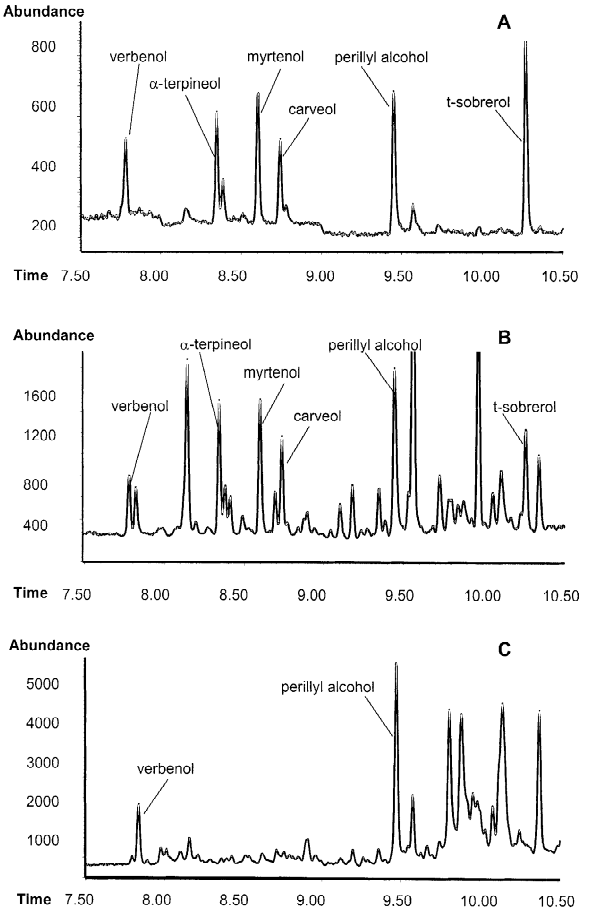

Fig. 2. GC–MS-chromatograms of (A) an external hydroxy terpene standard in methanol (each 50 μg/l), (B) an extract of a pooled urine spiked with hydroxy terpenes (each 25 μg/l), (C) an extract of an urine sample from a joiner.

droxide groups may be the reason for the partial sorption on the silica gel compared to the results without silica gel clean up (Tables 1 and 2). In contrast, the recovery rate of verbenol is improved by the clean up. Since it is difficult to find a suitable internal standard with the same physical–chemical characteristics that cannot be found in urine, external standard calibration with urine extracts of spiked urine samples was performed. The calibration function was linear in the concentration range from 1 to 1000 μg/l. The detection limits were calculated according to two different methods. The values determined by the signal-to-noise ratio are mostly lower than the values determined according to DIN 32645 [20] because a confidence interval is used by the latter method (significance level $\alpha=0.01$, $\beta=0.01$). Therefore the limits calculated according to DIN 32645 are more reliable. The detection limits (DIN 32645) of all hydroxy terpenes are in the range of 1.0 to 4.5 μg/l using 10 spiked urine samples in the concentration range of 0.5–5.0 μg/l. The value for *cis*-verbenol is significant lower than the value published to date using also GC–MS [14]. The background concentrations in the pool urine are for the most hydroxy terpenes in the same concentration range as the detection limits. Besides coeluting matrix compounds with similar mass fragments this resulted from the hydroxy terpenes itself because the urine of nonexposed persons can contain also terpene metabolites in low concentrations as the exposition with terpenes in indoor air and in food is ubiquitous. The standard deviation of the method is a parameter for the quality of the method and is valid over the

Table 3
Characteristic mass fragments and retention times of the hydroxy terpenes of the GC–MS method

	Retention time (min)	Mass fragments (m/z)	Assignment
cis-Verbenol	7.79	94	$M^+-CH_3-C_3H_7$
		109	$M^+-C_3H_7$
α-Terpineol	8.35	93	$M^+-H_2O-C_3H_7$
Myrtenol	8.60	84	$M^+-C_3H_6-C_2H_2$
		109	$M^+-C_3H_7$
Carveol	8.74	84	$M^+-C_3H_6-C_2H_2$
		109	$M^+-C_3H_7$
Perillyl alcohol	9.45	79	$M^+-C_3H_5-H_2O-CH_2$
		93	$M^+-C_3H_5-H_2O$
trans-Sobrerol	10.27	79	$M^+-CH_3-C_3H_6OH-OH$
		109	$M^+-H_2O-C_3H_7$

whole calibration range (0.5–5 μg/l) [20]. The calculated values are similar for all hydroxy terpenes except for perillyl alcohol that also showed the weakest detection limit (Table 2).

For evaluation the method was applied to a urine sample from a worker in a joiner's workshop (Fig. 2C). According to the high α-pinene concentrations measured in the air (43 $\mu g/m^3$), the urine contained the α-pinene metabolite verbenol in relatively high amounts (70 μg/l). The α-pinene metabolite myrtenol was not detected above the detection limit. But perillyl alcohol as limonene metabolite was determined in high concentration (124 μg/l). For clear identification of the peaks the sample was measured in the full scan mode of the MS detector, too, and the mass spectra of the peaks were compared with the MS spectra library.

4. Conclusions

The sample preparation and the developed GC–MS method allowed the simultaneous and sensitive determination of the urinary terpene metabolites *cis*-verbenol, α-terpineol, myrtenol, carveol, perillyl alcohol and *trans*-sobrerol. Low amounts of RP-C_{18}-SPE material and silica gel, as well as replacement of the evaporation step by elution with a small solvent volume enable an economic and quick sample preparation. Extraction and clean up by RP-C_{18}-SPE material and silica gel cartridge is essentially for a interference-reduced and sensitive GC–MS analysis. Sensitive biomonitoring of terpene exposure can be performed by the described method.

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