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Sensitive determination of monoterpene alcohols in urine by HPLC–FLD combined with ESI-MS detection after online-solid phase extraction of the monoterpene-coumarincarbamate derivates

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ABSTRACT

A method was developed for the determination of the monoterpene alcohols verbenol, myrtenol, perillyl alcohol, α-terpineol, Δ^3-carene-10-ol, thymol and *p*-α,α-trimethylbenzylalcohol in urine samples. After an enzymatic cleavage of their glucuronide- and sulfate conjugates the monoterpene alcohols were converted in the urine matrix with 7-diethylaminocoumarin-3-carbonylazide into monoterpene-[7-(diethylamino)-coumarin-3-yl]-carbamate derivates prior to analyses. Enrichment of the monoterpene alcohols from the urine matrix was achieved by online-solid phase extraction (SPE) with restricted-access material (RAM). After removal of excess derivatization reagent and urine matrix components, the monoterpene derivatives were separated by high-performance liquid chromatography (HPLC) in combination with fluorescence (FLD) detection and simultaneous mass spectrometric (MS) identification. Detection limits (LOD) for studied monoterpene alcohols ranged between 22 and 197 ng/L. The method was validated and successfully applied to urine samples from human subjects orally exposed to monoterpenes trough an intake of cough medication containing monoterpenes as active medicinal ingredients.

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1. Introduction

The monoterpenes α-pinene, β-pinene, camphene, Δ^3-carene, α-terpinene and limonene are naturally occurring compounds that are mainly released into the indoor air environment from building materials such as wood, paints and varnishes [1,2]. Monoterpenes are highly volatile compounds and they belong to the group of volatile organic compounds (VOCs) [3]. Beside to the naturally occurrence, monoterpenes and monoterpene mixtures are used as fragrance- and flavoring agents for foods, cosmetic products and cleaning agents [4] but are also natural ingredients of complementary- and alternative medicine (CAM) produced from natural raw materials (e.g. leaves, roots, etc.) [5].

In recent years a study reported that indoor air monoterpene concentrations were firmly increased, obviously due to a more frequently usage of natural products in the construction of houses, buildings and upholsteries [6]. Another previously published paper discussed the relationship between elevated (high) concentrations of α-pinene, β-pinene, Δ^3-carene and limonene and others [7] being responsible for causing occasionally irritation of skin, eyes and mucous membranes in humans. Prolonged exposure probably could also explain allergic contact dermatitis or chronic lung function impairment [8–12]. Occupational airway irritations were reported from exposure to monoterpenes in the mg/m^3 concentration range at mainly woodworking places [13–15]. Exposure to monoterpenes can be determined by biomonitoring of monoterpenes and/or monoterpene metabolites concentrations in biological fluids such as blood and urine from suspected exposed human subjects. The biomonitoring technique provides a very useful tool to estimate systemic uptake/exposure of man to monoterpenes and presents also an indication of the bioavailability of monoterpenes [16]. Previously, different studies reported the possibility of the determination of monoterpene systemic exposure by the analyses monoterpene alcohol concentrations from monoterpenes such as verbenol, a metabolite of α-pinene

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[17], 2α- and 3α-hydroxycineole [18] metabolites from 1,8-cineole and the Δ^3-carene metabolite, Δ^3-carene-10-ol [19] in urine samples. Good correlations between exposure to α-pinene and detected verbenol concentrations in urine from workers employed at joinery shops and sawmills were reported [13,15]. Systemic exposure of humans to monoterpenes was mainly studied by the application of gas chromatography–mass spectrometry (GC–MS) for determination of monoterpene and/or monoterpene metabolite concentrations in blood and urine samples from (suspected) exposed humans. Previously we published such a GC–MS method that allowed the determination of several monoterpene concentrations in urine [20]. However, we concluded that this method was not sensitive enough (LODs between 1.0 and 4.5 μg/L) to be able to determine the systemic uptake of monoterpenes by humans from breathing. Systemic uptake due to normal air inhalation had to be much lower then the lowest LOD of the method, so we were forced to develop a much sensitive and selective method. Liquid chromatography in combination with fluorescence and/or mass spectrometry seems to provide a tool to achieve higher sensitivity.

Besides the improvement of LODs possible automatization of the method was also very desirable. We decided that the approach of improvement of the LODs by derivatization of the monoterpene alcohols with fluorescence could be successful. Alcohols can be derivatized for HPLC–fluorescence analyses by reaction with carbonyl azides [21]. Saisho et al. [22] used previously successfully for example 7-methoxycoumarin-3-carbonylazide for the fluorescence detection of 7α-hydroxycholesterol and obtained a detection limit of 4 pg absolute. Until now derivatization of monoterpene alcohols was not yet applied as approach to reach higher sensitivity in human biomonitoring.

A few years ago, online purification and enrichment of analytes using solid phase extraction (SPE) with alkyl-diol silica, a restricted-access material (RAM) has been introduced in biomonitoring techniques. Alkyl-diol silica (ADS) belongs to internal surface reversed-phase support materials which are based like the conventional SPE adsorbent on silica but have a smaller pore size (6 nm) this in contrast to conventional silica which has a pore size of few micro meter. The molecular cut-off mass of used ADS in present paper was approx. 15,000 Da, macromolecules such as proteins, had no access to the inner surface of ADS and were therefore not retained. Low-molecular mass compounds like the monoterpene alcohol derivates are, penetrate into the pores and become retained on the RAM material. RAMs have been used in the past successfully in several applications especially with different biological fluids (e.g. haemolysed blood, plasma, serum, cell culture and tissue homogenates) to support the analysis of low-molecular weight compounds (e.g. drugs, metabolites, etc.) [23–25].

The aim of present study was the development of a new more sensitive method to determine and measure monoterpene alcohols in urine samples by means of HPLC with fluorescence detection after derivatization with a fluorogenic derivatization agent and simultaneous identification and confirmation by MS after electro spray ionization. In addition, we replaced the time-consuming and laborious manual solid phase extraction by an online-SPE using ADS material as adsorbent. The method was applied to a pilot study where healthy human subjects ingested a CAM containing monoterpenes as medicinal active ingredients to estimate systemic exposure to these monoterpenes by determination through the concentration of the monoterpene metabolites in collected urine samples during the pilot study.

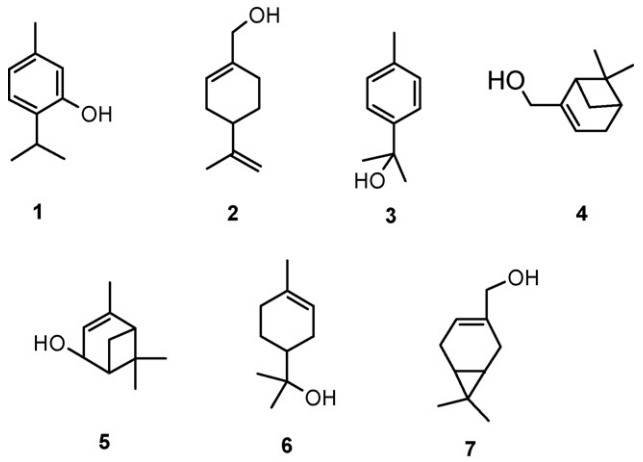

Fig. 1. Monitored monoterpene alcohols; (1) = thymol, (2) = perrilyl alcohole, (3) = *p*,α,α-trimethylbenzylalcohole, (4) = myrtenol, (5) = verbenol, (6) = terpineol and (7) = carenol.

2. Materials and methods

2.1. Chemicals

All chemicals used unless specified different were analytical grade quality and/or for biochemical use. Myrtenol (2-pinene-10-ol, MR) and perillyl alcohol (4-mentha-1,8-dien-7-ol, PA) were purchased from Fluka (Buchs, Switzerland), γ-terpineole (4-menth-1-en-8-ol, TP), thymol (2-isopropyl-5-methylphenol, TD), *p*,α,α-trimethylbenzylalcohol (1-methy-4-(1-hydroxy-1-methylethyl)benzene, TMD) and verbenol (2-pinene-4-ol, VB) were purchased from Sigma Aldrich (Taufkirchen, Germany). The derivatization agent 7-diethylaminocoumarin-3-carbonylazide (DACC) was purchased from Molecular Probes (Eugene, USA). The monoterpene alcohol Δ^3-carene-10-ol (CD) was synthesized in our laboratory as described previously [26]. Acetonitrile, methanol and toluene were purchased from Mallinckrodt-Baker (Deventer, The Netherlands) and the enzyme mixture β-glucuronidase/arylsulfatase was from Merck (Darmstadt, Germany). Water used for HPLC and preparation of solutions was prepared by a Milli-Q water purification system (Millipore, Eschborn, Germany). Chemical structures of all monoterpene alcohols are shown in Fig. 1.

2.2. Preparation of monoterpene-DACC standards

Primary stock solutions of all monoterpene-DACC derivatives (Fig. 2) were prepared by dilution of freshly synthetic prepared derivatives at a concentration of approx. 0.1 mg/mL in acetonitrile using following procedure: 200 μL of a solution containing of 7-diethylaminocoumarin-3-carbonylazide (16 μmol/mL) dissolved in a mixture of acetonitrile/toluene (1:1, v/v) were transferred into a 4 mL screw neck glass vial (45 mm × 14.7 mm) and 500 μL of a monoterpene alcohol solution (6.4 μmol/mL) dissolved in acetonitrile/toluene mixture were added. The vial was closed and heated for 120 min at a temperature of 85 °C. After this time, the reaction mixture was cooled down to room temperature and content was removed and diluted with acetonitrile to a precise volume of 10.00 mL. Synthetic recoveries and purity of the prepared monoterpene derivatives were determined by LC–FLD–MS procedure as described in Section 2.6.

2.3. Calibration curves

Calibration curves were prepared from the primary stock standard solutions (approx. 100 mg/L) synthesized as described in Section 2.2. From the stock standard solutions, a secondary

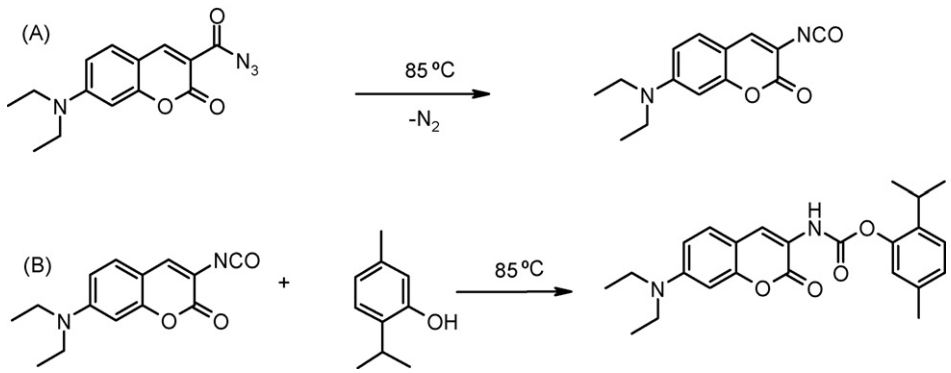

Fig. 2. Derivatization reaction of alcohols with DACC; (A) N_2 loss by an α-elimination reaction and Curtius rearrangement to 7-diethylaminocoumarin-3-isocyanate; (B) reaction of 7-diethylaminocoumarin-3-isocyanate with thymol to a 7-diethylaminocoumarin-3-yl-carbamate derivative of the monoterpene thymol.

standard stock solution containing 1000 ng/L of each monoterpene-DACC derivate was prepared by serial dilution of the stock standard solutions with pooled control urine collected from non-exposed human subjects ($n = 3$). Serial dilutions of this secondary stock standard solution with pooled control urine were made to obtain standard curve points of 10, 30, 50, 70, 90, 110, 130, 150, 170, 190, 210, 230 and 250 ng/L in 20 mL pooled control urine.

2.4. Enzymatic hydrolysis of urine samples

Pooled urine samples collected from exposed human subjects were enzymatic hydrolyzed with an enzyme mixture (Merck, Darmstadt, Germany) containing β-glucuronidase (EC 3.2.1.31, 30 U/mL) and arylsulfatase (EC 3.1.3.1, 60 U/mL) both obtained expressed in *Helix pomatia*. Therefore, 20 mL from the urine samples of exposed human subjects (see Section 2.8) and 20 mL of pooled control urine were mixed with 10 mL of a sodium acetate (NaAC) solution (pH 5.0, 0.1 M). The pH of the solution was adjusted to pH 5.0 with a diluted aqueous hydrochloric acid solution (HCl, 0.05 M) and 25 μL of the enzyme mixture were added and samples were shaken and incubated at a temperature of 37 °C for approx. 24 h as described previously by Sandner et al. [20]. The enzymatic hydrolysis reaction was stopped by addition of 1.6 mL of pure methanol to the solutions.

2.5. Derivatization and online-solid phase extraction

Derivatization of the hydrolyzed urine samples (Section 2.4) was carried out prior to online extraction. Therefore 100 μL of a DACC solution (0.32 mmol/L) dissolved in acetonitrile was added to the hydrolyzed urine samples (Section 2.4) and solutions were heated for 120 min at a temperature of 85 °C. After the derivatization procedure, urine samples were cooled down to room temperature on the laboratory table. Furthermore, 10 μL of the derivatized urine hydrolysate samples were injected by the autosampler and transferred onto the SPE trap column (Lichrospher RP-ADS, 5 μm, 125 mm × 4 mm I.D., Merck, Darmstadt, Germany) by a System Gold 126 pump (Beckmann, Germany).

At the start of the online-SPE procedure, the 2-position-10 way valve was set to position 1 (Fig. 3A) while the Gold 126 pump (sample transfer pump) was set to a flow of 0.05 mL/min (acetonitrile/water; 70%/30%) so that analytes were transferred onto the SPE trap column and quantitative trapped. During method development we observed the elution of the urine matrix from the SPE column by fluorescence detection (Fig. 3A, connection between 5 and the FLD detector). Later on during trapping of the samples the matrix was directly transferred to the waste.

Injection volume of the samples tested varied between 10 and 50 μL resulting in injections of 10 μL because of best chromatographic separation and peak shape.

2.6. Liquid chromatography with MS- and FLD detection

After the trapping/enrichment of derivatized urine samples onto the trap column, the pumps of the HPLC system and HPLC gradient were started automatically. The 2-postion-10 way valve was after 12.5 min switched to position 2 (Fig. 3B) and the valve was hold in this position for consecutive 14 min and analytes were transferred onto the analytical HPLC column. After a total processing time of

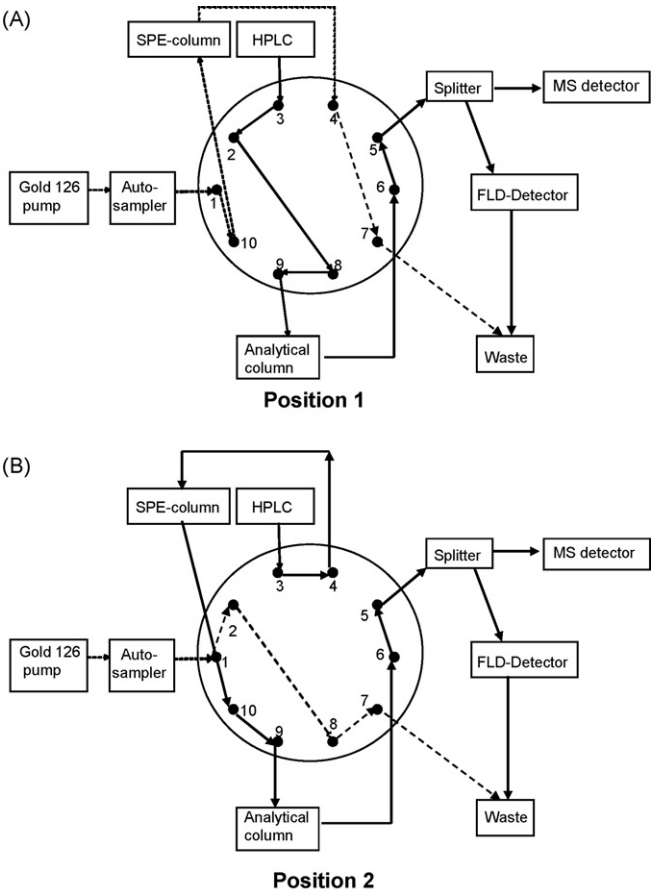

Fig. 3. Illustration of the 2-position-10 way valve used for online-SPE application. (A) Position 1; during online-SPE; (B) Position 2; during HPLC/FLD–MS analysis.

Table 1
Preparative obtained synthetic recoveries and purities of monoterpene-[7-(diethylamino)-coumarin-3-yl]-carbamate derivatives as illustrated in Figs. 1 and 2

Monoterpene metabolite	Derivative	Derivative abbreviation	Molecular ion $[M+H]^+$	Synthetic recovery (%)	Purity (%)
Thymol (1)	(2-Isopropyl-5-phenyl)-[7-(diethylamino)-coumarin-3-yl]-carbamate	TD	409.2	91	100
Perillyl alcohole (2)	(*p*-Mentha-1,8-dien-7-yl)-[7-(diethylamino)-coumarin-3-yl]-carbamate	PA	411.2	93	98
p,α,α-Trimethylbenzyl alcohole (3)	(*p*,α,α-Trimethylbenzylalkyl)-[7-(diethylamino)-coumarin-3-yl]-carbamate	TMD	409.2	87	98
Myrtenol (4)	(2-Pinene-10-yl)-[7-(diethylamino)-coumarin-3-yl]-carbamate	MR	411.2	89	96
Verbenol (5)	(2-Pinene-4-yl)-[7-(diethylamino)-coumarin-3-yl]-carbamate	VB	411.2	80	88
Terpineol-8-ol (6)	(*p*-Menth-1-en-8-yl)-[7-(diethylamino)-coumarin-3-yl]-carbamate	TP	413.2	59	92
Δ^3-Carene-10-ol (7)	(Δ^3-Carene-10-yl)-[7-(diethylamino)-coumarin-3-yl]-carbamate	CD	411.2	84	95

26.5 min the valve was switched back to position 1 (Fig. 3A) and hold in this position for another 7 min before a new sample was injected. The analytes were separated by gradient elution, eluent A consisted of water with 0.5% acetic acid and eluent B was 100% acetonitrile, respectively. The separation was achieved by following gradient (0 min: 70% B: 2 min 70% B: 17 min 100% B: 30 min 90% B and 33 min 70% B and 35 min 70% B) at a flow of 0.4 mL/min and column temperature of 40 °C. Separation of the monoterpene-DACC derivatives was accomplished using a Gemini C_{18} HPLC column (250 mm × 2.0 mm, 5 μm, Phenomenex, Germany).

The eluent coming from the HPLC column was divided by a flow splitter (Valco, Germany) before introduction into the MS system and fluorescence detector. 125 μL/min were introduced into the mass spectrometer and 275 μL/min were directed to the fluorescence detector. The fluorescence detector was set to a combination of an excitation wavelength of 376 nm and emission wavelength of 480 nm. The mass spectrometer used was a SSQ 7000 single quadrupole mass spectrometer (Finnigan, Hamburg, Germany) using positive electro spray ionization monitoring ions from m/z = 100–800 Da. Maximum sensitivity of the MS system was obtained at following instrument settings; heated capillary temperature: 200 °C, spray voltage: 4.5 kV, scan time 1 s, conversion dynode: 15 kV, electron multiplier voltage 1650 V, sheath gas pressure (N_2): 0.45 Pa and auxiliary gas (N_2) pressure: 0.10 Pa.

2.7. Method validation

The method was validated by determination of limit of determination (LOD) using a signal/noise ratio (S/N) of 3:1 and limit of quantization (LOQ) using S/N of 10:1 for each monoterpene-DACC derivative. Precision of the method was determined by analyzing validation samples prepared from pooled control urine spiked with monoterpene-DACC derivatives at two different concentration levels of 200 and 500 ng/L (n = 3). Accuracy of the analytical method was determined by the method's intra- and interday accuracy using validation samples spiked at 200 and 500 ng/L (n = 3).

2.8. Application of the method to real urine samples of exposed human subjects

Two overtly healthy human subjects were enrolled in a small trial where subjects voluntary swallowed during a consecutive period of five days a CAM called Gelomyrtol® forte (Pohl-Boskamp, Hohenlockstedt, Germany). The CAM contained as active medicinal ingredients monoterpenes. The CAM was given as tablets (300 mg, 3–4 tablets/day) containing each 75 mg of limonene and cineole and 20 mg of α-pinene, respectively. This CAM was purchased in a drugstore and is in Germany freely available without a doctor's recipe and is frequently used in the treatment of acute or chronic bronchitis and sinusitis. After the orally intake, urine of one day was sampled and pooled and then frozen into plastic containers and stored at a temperature of −20 °C until sample preparation and analyses as described in Sections 2.5 and 2.6.

3. Results and discussion

3.1. Development and validation of method

Prepared DACC derivatives from organic synthesis had satisfying synthetic recoveries (recovery >80% theoretical) as well high enough purity (purity 80–100%) required for validation of the new developed method (Table 1). Exception was the DACC derivative of terpineol-8-ol with a recovery rate of 59%. This low recovery was explained by the fact that ternary alcohols contain no α-hydrogen atoms and will therefore negatively influence the synthetic recovery, this in contrary to primary- and secondary alcohols which contain α-hydrogen atoms.

All monoterpene-DACC derivatives could be trapped on an online-SPE column with the special RAM adsorbent material. Before completely establishment of the method, monoterpene-DACC derivatives were analyzed with different HPLC and MS conditions (data not shown) until optimal liquid chromatographic and mass spectrometric settings were obtained. Fluorescence

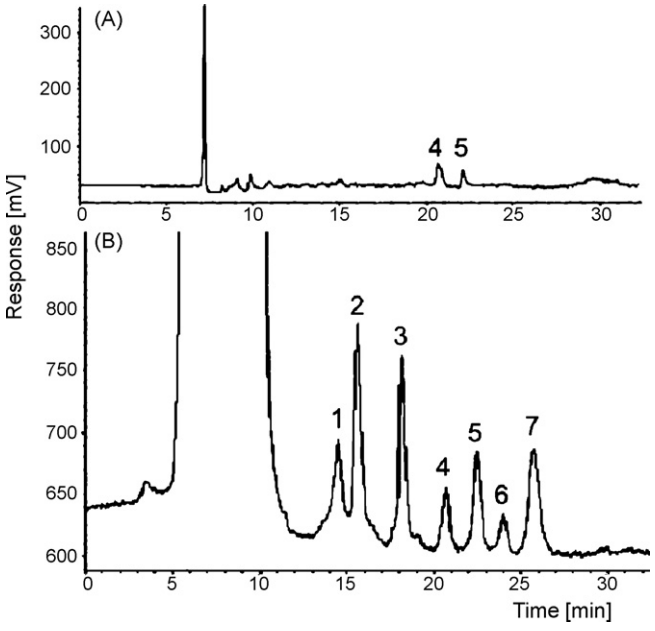

Fig. 4. HPLC–FLD chromatogram of a hydrolyzed urine sample from an exposed human subject to GeloMyrtol (A) containing two monoterpene alcohol-DACC derivatives (4 and 5); (B) chromatogram of a spiked pooled urine sample (50 μg/L) containing seven monoterpene alcohol-DACC derivatives thymol, R_t = 14.7 min (1); perrilyl alcohole, R_t = 15.8 min (2); *p*,α,α-trimethylbenzylalcohole, R_t = 18.4 min (3); myrtenol, R_t = 20.4 min (4); verbenol, R_t = 22.5 min (5); terpineol, R_t = 23.9 min (6) and carenol, R_t = 25.7 min (7).

Table 2
Limits of detection- and quantification

Monoterpene alcohol[a]	Regression coefficient	LOD	LOQ
	r^2	ng/L	ng/L
Thymol	0.9993	23	67
Perillyl alcohole	0.9994	22	47
p,α,α-Trimethylbenzylalcohole	0.9926	95	237
Myrtenol	0.9971	101	216
Verbenol	0.9915	55	111
Terpineol	0.9906	197	476
Δ^3-Carene-10-ol	0.9985	52	106

[a] As monoterpene-[7-(diethylamino)-coumarin-3-yl]-carbamate.

wavelengths were determined by scanning single DACC derivatives in the scan facility mode, maximum excitation- and emission wavelengths were selected and used. Fig. 4B depicts a representative fluorescence chromatogram for 50 μg/L of each monoterpene-DACC derivative extracted from spiked pooled control human urine. During method development the elution of the urine matrix was observed by fluorescence detection. The high fluorescence signal shows the elution during 5 and 12 min. Later on the eluate of the trap was during 12.5 min directly transferred to the waste to avoid contamination of the detector.

One important issue of the new developed method was automatization of the method by installation of a 2-position-10 way valve. The switching times of the valve was influenced by the retention times of the monoterpene-DACC derivatives. Breakthrough time (volume) of the analytes (t_a), complete elution of the urine matrix components from the trap column (t_m), complete elution of the analytes from the trap column (t_t) were determined and the point that the valve had to be switched back to the start position (t_{v1}) enabling trapping of a new sample and the time that the valve had to be switched to transfer the trapped monoterpene-DACC derivatives from the trap column onto the analytical column (t_{v2}) was also determined. Experiences and results obtained from previous experiments with automated enrichment and purification of samples led to the formation of the empirical Eqs. (1)–(3) that allowed us the determination of the switching times for the 2-postion-10 way valve;

$$t_{v1} = t_m + 1 \text{ min if } t_a > 10 \text{ min and} \quad (1)$$

$$t_{v1} = 0.5 * (t_m + t_a) \text{ if } t_a < 10 \text{ min} \quad (2)$$

$$t_{v2} = t_t + 1 \text{ min} \quad (3)$$

t_a was found to be >10 min so t_{v1} was calculated by Eq. (1) and was set to 12.5 min resulting in a quantitative online purification and extraction of monoterpene-DACC derivatives.

After the last analyte eluted from the trap column the 2-postion-10 way valve had to be switched to position where the trapped analytes could be transferred to the analytical column to be separated, this was done at 1 min after the last analyte eluted (t_t).

Then calibration curves were determined in the concentration range between 10 and 250 ng/L and calibration curves were constructed by plotting calculated area of the monoterpene-DACC calibrators (Section 2.3) versus their known concentrations. The calibration curves were used to determine the monoterpene-DACC concentrations in real urine samples. Calibration curves were calculated using linear regression without weighing. The correlation coefficients (r^2) for selected concentration range were >0.99 for all monoterpene-DACC derivatives (Table 2).

The accuracy and precision of the method was investigated by both intra- and interday experiments and were validated from three replicates of validation samples of known spiked monoterpene-DACC concentrations (200 and 500 ng/L) prepared in pooled control human urine. The precision of the method is expressed as the relative standard deviation calculated from multiple replicate measurements from these spiked validation samples. All obtained intra- and interday precision and accuracy values were acceptable and spanned the concentration range until 500 ng/L (Table 3).

The LOD of each monoterpene-DACC derivative analyzed with the fluorescence detection method was determined at a signal/noise ratio (S/N = 3, n = 3) and were between 22 and 197 ng/L while LOQs (S/N = 10, n = 3) were between 47 and 476 ng/L, respectively (Table 2). The obtained LODs and LOQs of presented method in this paper are approx. one hundred times lower than the GC–MS method developed in the past for the determination of monoterpene alcohols in urine samples [20] making the method applicable for the measurement of very low concentrations in systemic exposure studies.

3.2. Analysis of real urine samples

After validation of the method it was successfully applied to the determination of monoterpene metabolites in pooled human urine from healthy volunteers exposed to monoterpenes. A reprehensive HPLC–FLD chromatogram of urine samples from a voluntary exposed subject is shown in Fig. 4A while the chromatogram of a spiked pooled urine sample (50 μg/L) is illustrated in Fig. 4B. Two metabolites of the monoterpene α-pinene were identified in the urine sample of exposed human volunteers. The first peak (#4) in the chromatogram was identified as Myrtenol (4) with a mean concentration of 30 ± 5 μg/L (mean ± S.D., n = 2) while the second peak (#5) was identified as verbenol (5) with a mean concentration of 46 ± 4 μg/L (mean ± S.D., n = 2).

Metabolites in the pooled urine samples from the exposed human volunteers were quantified by HPLC–FLD analysis and metabolite identity can be confirmed by LC–MS analysis. In Fig. 5A and B typical mass spectra of verbenol- and myrtenol derivatives are shown.

Table 3
Intraday- and interday validation accuracy and precision for all monoterpene-[7-(diethylamino)-coumarin-3-yl]-carbamate derivatives in pooled control human urine (3 replicates per day)

Derivative	TD		PA		TMD		MR		VB		TP		CD	
	Intraday validation													
Spiked concentration (μg/L)	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50
Mean concentration (μg/L) measured	0.21	0.48	0.19	0.51	0.18	0.51	0.21	0.48	0.21	0.51	0.23	0.45	0.19	0.52
Accuracy (ng/L)	7.0	11.0	6.2	12.5	8.6	15.2	8.5	14.5	7.8	14.0	9.2	21.0	8.0	13.0
Precision (%)	4.0	7.5	2.5	2.0	7.5	3.0	7.5	4.0	3.0	2.5	15.0	10.0	5.0	4.0
	Interday validation													
Spiked concentration (μg/L)	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50	0.20	0.50
Mean concentration (μg/L) measured	0.21	0.49	0.20	0.51	0.19	0.51	0.21	0.49	0.19	0.51	0.22	0.46	0.21	0.52
Accuracy (ng/L)	7.2	11.5	6.0	12.5	9.4	14.5	8.2	14.0	7.4	13.5	9.5	19.0	7.8	13.2
Precision (%)	5.0	2.0	2.5	2.0	5.0	2.0	5.0	2.0	2.0	2.5	10.0	8.0	5.0	4.0

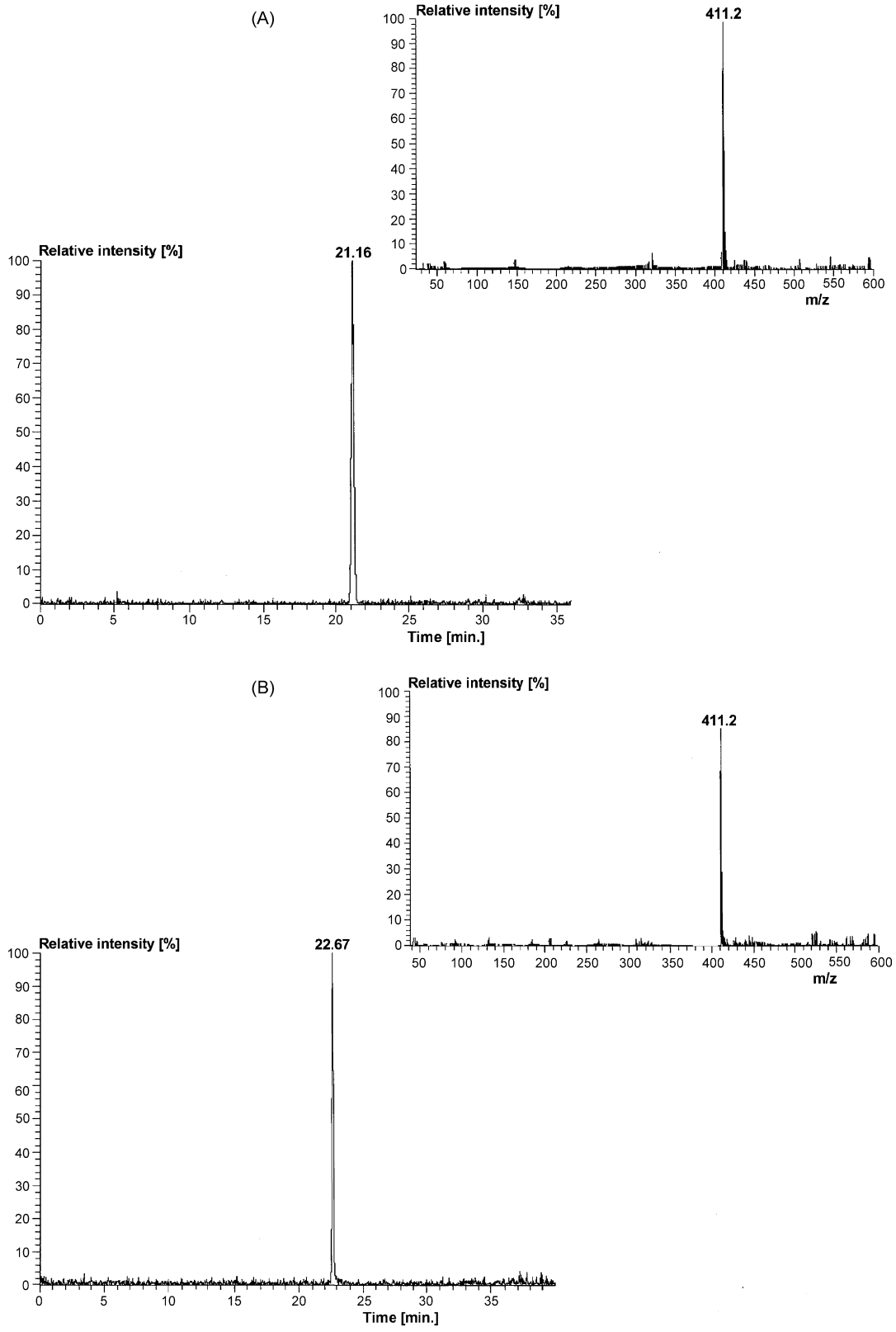

Fig. 5. (A) Retention time and mass spectrum from the monoterpene-DAC derivative MR (4) from Fig. 4B. (B) Retention time and mass spectrum from the monoterpene-DACC derivative VB (5).

4. Conclusion

The determination of monoterpene alcohols in urine led to a newly selective, accurate, precise and robust HPLC method with fluorescence and mass spectrometric determination of monoterpene alcohols in urine. The new method is in contrary with the previously published method [18] much more sensitive. The method provides an acceptable chromatographic resolution of the monoter-

pene alcohols with excellent limits of detection and quantification. The method was validated over the concentration range from 10 to 500 ng/L for all monoterpene alcohols. The method has also been successfully applied to urine samples from healthy volunteers exposed to monoterpenes by an orally intake of a CAM with three different monoterpenes as active ingredients.

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