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Separation of attogram terpenes by the capillary zone electrophoresis with fluorometric detection

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ABSTRACT

An original method based on capillary zone electrophoresis with fluorimetric detection has been developed for the determination of terpenic compounds. The method is based on the separation of a terpenes dynamically labeled by the non-ionogenic tenside poly(ethylene glycol) pyrenebutanoate, which was used previously for the labeling of biopolymers. The background electrolytes were composed of taurine–Tris buffer (pH 8.4). In addition to the non-ionogenic tenside aceton and poly(ethylene glycol) were used as the additives. The capillary zone electrophoresis with fluorometric detection at the excitation wavelength 335 nm and the emission wavelength 463 nm was successfully applied to the analysis of tonalid, cholesterol, vitamin A, ergosterol, estrone and farnesol at level of 10^{-17} mol L^{-1}. Farnesol, is produced by *Candida albicans* as an extracellular quorum-sensing molecule that influences expresion of a number of virulence factors, especially morphogensis and biofilm formation. It enables this yeast to cause serious nosocomial infections. The sensitivity of this method was demonstrated on the separation of farnesol directly from the cultivation medium.

1. Introduction

Terpenes are a large and varied class of hydrocarbons which are produced by a wide variety of plants and by some animals [1]. In plants, they are known to have mainly ecological roles in acting as deterrents against feeding by herbivores, as antifungal defenses and attractants for pollinators [2]. In mammals, terpenes are involved in stabilization of cell membranes, in metabolic pathways and act as regulators of enzymatic reactions [3]. Basic properties of most terpenes are their volatility, the low concentrations in samples, they are nearly insoluble in water but well soluble in common organic solvents.

Gas chromatography (GC) [4] for volatile terpenes and high performance liquid chromatography (HPLC) [5–8] are the most common methods for routine detection and identification of terpenes. They are often associated with mass spectrometry (MS) [9–22]. Chen et al. [23] studied polycyclic musks by GC–MS in the air, wastewater and sludge samples of a typical cosmetic plant. Tonalid, as a representant of polycyclic musks group, is one of the most commonly used fragrance ingredient. The limit of detection (LOD) was calculated for tonalid at ng L^{-1} by GC–MS [23,24]. Farnesol has been detected among volatile compounds of wines after stir bar sorptive extraction and liquid desorption combined with large volume injection GC quadrupole MS [25]. Also combination GC–GC analysis has been used to separate complex mixtures of enantiomeric monoterpene hydrocarbons and alcohols in bergamont oils [26].

HPLC analysis offers some advantages compared to GC and can be carried out when GC analysis of thermolabile and/or polar compounds is difficult to achieve. Nonvolatile materials have to be handled or preparative isolation of volatile compounds are required [27]. Yuan et al. [5] separated and detected 0.01 mg L^{-1} of ergostetrol by HPLC. Using this method, the UV detection limit for vitamin A was reached in the range of attomol L^{-1} [28]. Mondello et al. [29] separated aliphatic monoterpene and sesquiterpene aldehydes from sweet orange oil by HPLC in connection with high resolution GC (HRGC). Ergosterol, 0.015 mg L^{-1}, was also detected by supercritical carbon dioxide extraction and ultraperformance liquid chromatography at atmospheric pressure by chemical ionization-mass spectrometry [30]. Detection limit was reached for estrone as 0.4 fg L^{-1}, by HPLC–tandem mass spectrometry (HPLC–MS/MS) [10].

Capillary zone electrophoresis (CZE) is a separation technique that can offer an alternative to GC and HPLC methods. The most attractive CZE advantages are minimum sample and reagent requirement, high resolution and short analysis time. Steroids [31] including estrone [32] were already separated using CZE as well

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as cholestrol [33], farnesol [34] and anothers terpenes [35,36]. The detection limit ranges from $\mu g\,L^{-1}$ to $pg\,L^{-1}$. Ma et al. [37] developed CZE technique for the minimicroassay of vitamin A in human serum. The detection limit was 10 fg of vitamin A at a signal-to-noise of 5:1 for this technique.

Since the terpenes have drawn increasing commercial attention due to understanding of their roles in prevention and therapy of several diseases, their activity as natural insecticides and antimicrobial agents [2], we decided to develop a method for the trace analysis. Recently, the non-ionogenic tenside based on pyrenebutanoate, poly(ethylene glycol)4-(1-pyrene)-butanoate (PB-PEG), was used for the labeling of the low concentrated biopolymers at CZE separation with fluorimetric detection [38,39]. In spite of the hydrophobic properties of terpenes we applied this CZE method for the separation of tonalid, cholesterol, ergosterol, vitamin A, estrone and farnesol, see Table 1. We assume, that the principle of tagging these small molecules is different from the labeling of biopolymers [38,39]. Most likely, small molecules migrate incorporated in the center of hydrophobic cavities of micelles to the detector. The labeling reaction of terpenes must proceed rapidly, as well as in the analysis of bioanalytes and the labeling reagent must have a low fluorescence in the unbound state and a high fluorescence enhancement when bound to analytes [38].

Farnesol was identified as a quorum-sensing molecule (QSM) in *Candida albicans* (*C. albicans*) which is the most common fungal pathogen in human and it is a causative agent of serious nosocomial infections, particularly bloodstream infections [40,41]. When farnesol accumulates above a threshold level, specific receptors are activated and it leads to coordinated gene expression [42]. A total of 274 genes were identified as responsive, especially hyphal formation-associated genes, the genes with roles related to cell surface hydrophobicity drug resistance, cell wall maintenance and iron transport [43]. It controls a number of *C. albicans* virulence factors, e.g., biofilm formation and morphogenesis (the morphological switch from the yeast to the filamentous form) [44]. Established detection of farnesol can help to understand the pathogenesis of *C. albicans* infection and biofilm formation. Moreover, it is thought to be a novel target for the development of drugs intended to prevent biofilm production by *C. albicans*. Therfore we chose farnesol as an example of practical application.

2. Experimental

2.1. Chemicals

The buffer component tris(hydroxymethyl)aminomethane, Tris, was obtained from Sigma (St. Louis, MO, USA), taurine, tonalid, farnesol and 4-(1-pyrene)butyric acid were from Aldrich (Milwaukee, WI, USA), estron from Merk (Darmstadt, Germany), cholesterol pure and ergosterol pure from Pliva-Lachema (Brno, Czech Republic), vitamin A acetate (2 800 000 IU/g) from Koch-Light Laboratories (Colnbrook Bucks England) and poly(ethylene glycol) (PEG – Mw 10 000) from Fluka (Buchs, Switzerland). Poly(ethylene glycol) pyrenebutanoate was synthesized in the Institute of Analytical Chemistry Academy of Sciences of the Czech Republic, v.v.i., Brno [45].

2.2. Cultivation, condition and composition of media

The strain included in this study, *C. albicans* CCM 8180, was obtained from Czech Collection of Microorganisms (Brno, Czech Republic). Before each experiment, the strain was cultivated on Sabouraud dextrose agar (HiMedia, Mumbai, India) at 37 °C for 24 h. The yeast culture was resuspended in physiological saline solution (PSS) to the density equal to McFarland 1 standard (3×10^{11} cells L^{-1}). Batches of Difco yeast nitrogen base medium (YNB, Becton-Dickinson, San Jose, CA, USA) containing 50×10^{-3} mol L^{-1} glucose (10 mL in 50 mL Erlenmeyer flasks) were inoculated with 100 μL of the yeast cell suspension and incubated at 37 °C in an orbital shaker at 300 rpm. Yeast cells were removed from the medium by centrifugation (1500 rpm for 10 min) and the culture supernatants were stored in glass vials at −20 °C.

2.3. Safety

Microorganisms, *C. albicans*, from a risk group 2 infectious agents was analyzed in this study. This yeast is unlikely to be seriously hazardous to laboratory personnel. Laboratory exposures rarely cause an infection leading to a serious disease; an effective treatment and preventive measures are available, and the risk of spreading is limited. Therefore, a biosafety level 2 is necessary for safe handling.

2.4. Instrument

The capillary zone electrophoretic experiment was carried out using the laboratory-made apparatus [46] with a constant voltage (−) 20 kV applied on the side of the detector and supplied by high voltage unit Spellman CZE 1000 R (Plainview, NY, USA). The lengths of the fused silica capillaries (FS), 0.05 mm I.D. and 0.25 mm O.D. (Pliva-Lachema), were 200 mm to the detector, with the column effective volumes of ∼1.6 μL. The both ends of the fused silica capillary were dipped in 3 mL glass vials containing the electrodes and background electrolytes (BGEs) of CZE. For the fluorimetric detection, the PU4027 Programmable Fluorescence detector (Philips Scientific, Cambridge, Great Britain) was modified. The excitation wavelength, λ_{EX}, was 335 nm, and the emission wavelength, λ_{EM}, was 463 nm [38,40]. The detector signals were acquired and processed with the Chromatography data station Clarity (DataApex s.r.o., Praha, Czech Republic). The ultrasound bath Sonorex, Bandelin electronic (Berlin, Germany) was used to samples as prevention absorption on wall of vials.

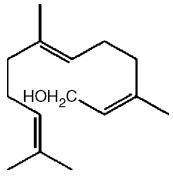
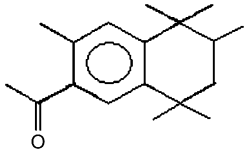
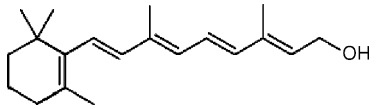
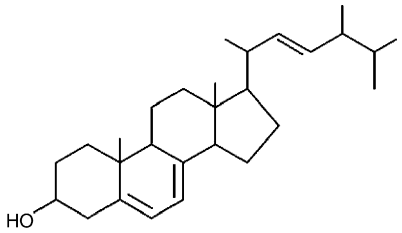
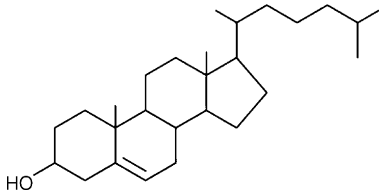
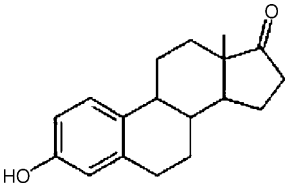
2.5. CZE conditions

The BGE was composed of 3×10^{-3} mol L^{-1} taurine–Tris buffer (pH 8.4), 5×10^{-5} to 7×10^{-5} mol L^{-1} PB-PEG, from zero to 20% (v/v) acetone and 0.6% (w/v) PEG 10000. Between the measurements, the capillary was washed with acetone/ethanol mixture (10:1, v/v) for 5 min and then backflushed with BGE for 2 min. The rinsing procedures were carried out hydrodynamically for 8–12 s. The height difference of the reservoirs for the sample injection, Δh, was 100 mm, the time of injection, $t_{inj.}$, was from 8 to 12 s. The injected volumes of the analytes were approximately 30–40 nL. The sample was dissolved in 5.0×10^{-5} to 1.3×10^{-4} mol L^{-1} PB-PEG and buffer 3×10^{-3} mol L^{-1} taurine–Tris. 1% ethanol was added in case of farnesol. The incubation time of the samples in the solution of the non-ionogenic tenside PB-PEG was 10 min at the laboratory temperature 25 °C. The samples were then immediately analyzed. The concentrations of the terpenes were in the range 1×10^{-16} to 1×10^{-19} mol L^{-1}. Before each measurement, the sample was sonicated for 2 min at the temperature of 25 °C and with the frequency of 35 kHz.

3. Results and discussion

Terpenes have different physical (small molecules, easy structures, see Table 1) and chemical properties (high hydrophobicity) compared to biopolymers. Different experimental conditions were necessary to use for the CZE separation of the examined terpenes

Table 1
Analyzed substances.

Farnesol 222.37 g mol^{-1}		Tonalid 258.398 g mol^{-1}	
Vitamín A 286.456 g mol^{-1}		Ergosterol 396.65 g mol^{-1}	
Cholesterol 386.65 g mol^{-1}		Estrone 270.366 g mol^{-1}	

with fluorometric detection in comparison with trace analysis of labeled biopolymers [45]. According our preliminary experiments biopolymers are partially or fully adsorbed onto the inner surface of the separation capillary in dependence on their concentration in the sample pulse at the conditions used for the separation of terpenes. Therefore, practical implementation of this new method of labeling could be performed for small hydrophobic molecules on farnesol and extracellular product formation of the biofilm at *C. albicans*.

3.1. CZE of farnesol

First, the experimental conditions were optimized for the separation of farnesol, which structure is the simplest from the examined terpenes. In the individual CZE runs, the PB-PEG concentration was optimized in the sample as well as in BGE. According to our preliminary experiments, the possible sample concentration may vary for farnesol from 10^{-17} to 10^{-18} mol L^{-1}. In this concentration range of farnesol the PB-PEG concentration in BGE has a significant effect on the peak shape, see Fig. 1A, different from the concentration of PB-PEG in the sample. For these experiments the taurine–Tris buffer, pH 8.4, was composed from 0.5×10^{-5} to 7.5×10^{-5} mol L^{-1} PB-PEG and 0.6% (w/v) PEG 10000. PEG 10000 was added into the BGE as an additional prevention of the terpenes adsorption onto the inner surface of the capillary. Concentration of 10^{-17} mol L^{-1} farnesol was dissolved in BGE with 5×10^{-5} mol L^{-1} PB-PEG. The incubation time of the samples in the solution of PB-PEG was found as 10 min.

Generally, when BGE was without adition of PB-PEG, farnesol was not detected because the PB-PEG majority from the sample was adsorbed onto the inner surface of the capillary. If PB-PEG was in an excess in BGE, a farnesol peak was not detected as well due to the high detection of the background response. At these used conditions the optimal concentration was found for PB-PEG in BGE, as well as in the sample, as 5×10^{-5} mol L^{-1}. The results of the optimizations shows narrow peak of farnesol in electropherogram in Fig. 1B. The increase PB-PEG concentration in the sample influenced the area of the first peak PB-PEG residue, migrant by the velocity of the electroosmotic flow. At these conditions a calibration

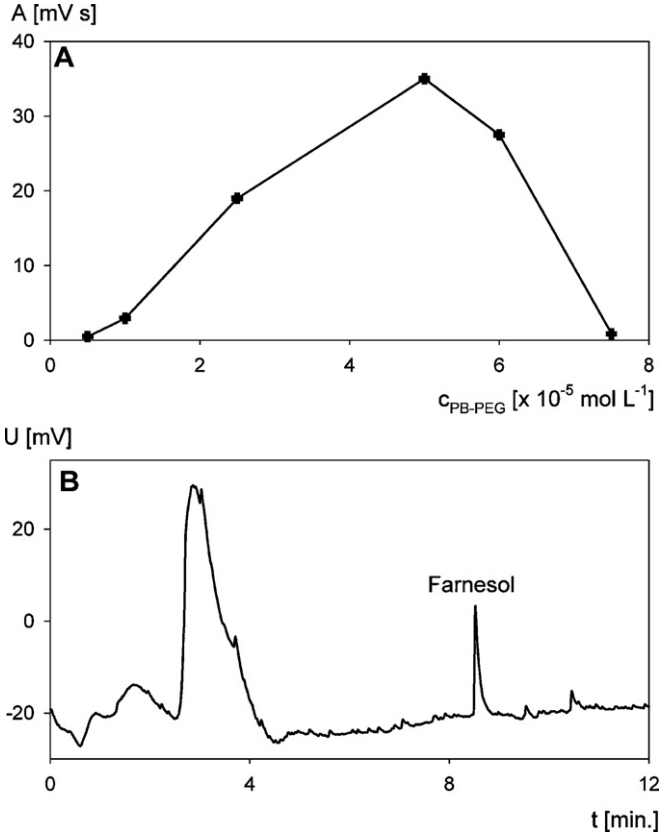


Fig. 1. Optimization procedures: (A) dependence of the peak area A [mV s] on the concentration of PB-PEG in BGE, $C_{PB\text{-}PEG}$ [$\times 10^{-5}$ mol L^{-1}]; (B) CZE separation of farnesol dynamically modified by PB-PEG. Conditions, FS, 0.05 mm I.D. and 0.25 mm O.D., length 400 mm, 200 mm to the detection cell; applied voltage (−) 20 kV, λ_{EX} 335 nm, λ_{EM} 463 nm; BGE composition, 3×10^{-3} mol L^{-1} taurine–Tris buffer (pH 8.4), 0.5×10^{-5} to 7.5×10^{-5} mol L^{-1} PB-PEG, 0.6% (w/v) PEG 10000; sample composition, 1×10^{-17} mol L^{-1} farnesol dissolved in buffer solution with 5×10^{-5} mol L^{-1} PB-PEG and 1% (v/v) ethanol, B see A, $C_{PB\text{-}PEG}$, 5×10^{-5} mol L^{-1} PB-PEG.

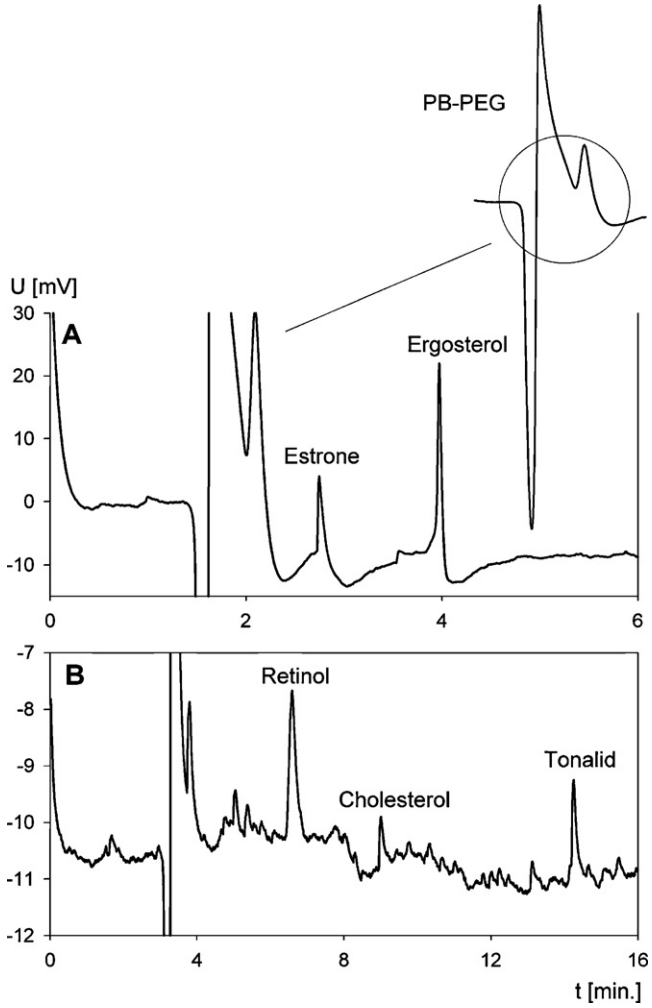


Fig. 2. CZE of dynamically modified estron, ergosterol (A) and tonalid, vitamin A and cholesterol (B) by PB-PEG. Conditions, see Fig. 1; BGE composition, 3×10^{-3} mol L^{-1} taurine–Tris buffer (pH 8.4), 7×10^{-5} mol L^{-1} PB-PEG, 20% (v/v) aceton and 0.6% (w/v) PEG 10000; sample composition, estron and ergosterol, each 5×10^{-19} mol L^{-1}, 1.3×10^{-4} mol L^{-1} PB-PEG dissolved in buffer solution. B, see A; sample composition, tonalid, vitamin A and cholesterol, 1×10^{-16} mol L^{-1} each dissolved in buffer solution of 7×10^{-5} mol L^{-1} PB-PEG.

curve was measured as a dependence of farnesol peak area on its concentration in the range from 1.3×10^{-17} to 1.6×10^{-18} mol L^{-1}. The calibration curve yielded a correlation coefficient of 0.99, the number of measurement was $n = 5$. RSDs of the migration times for farnesol are lower than 2% and RSDs its peak areas are lower than 4%. The minimum detectable farnesol amounts were achieved in attograms.

3.2. CZE of more complex terpenes

Under conditions used for CZE of farnesol, the other terpenes, see Table 1, were fully adsorbed onto the inner capillary surface, because they are more hydrophobic. The suitable PB-PEG concentrations and additives were necessery to find in both sample and BGE. The 3×10^{-3} mol L^{-1} taurine-Tris buffer solution was used as BGE at pH 8.4 with 20% (v/v) aceton, 0.6% (w/v) PEG 10000 and PB-PEG, whose concentrations varied depending on identifying terpenes. Estrone and ergosterol, 5×10^{-19} mol L^{-1}, was dissolved in BGE with 1.3×10^{-4} mol L^{-1} of PB-PEG. The electropherogram of this separation is depicted in Fig. 2A. Unbound PB-PEG in the sample migrates by the velocity of the electroosmotic flow – the detail of the first peak in Fig. 2A like as in Fig. 1B.

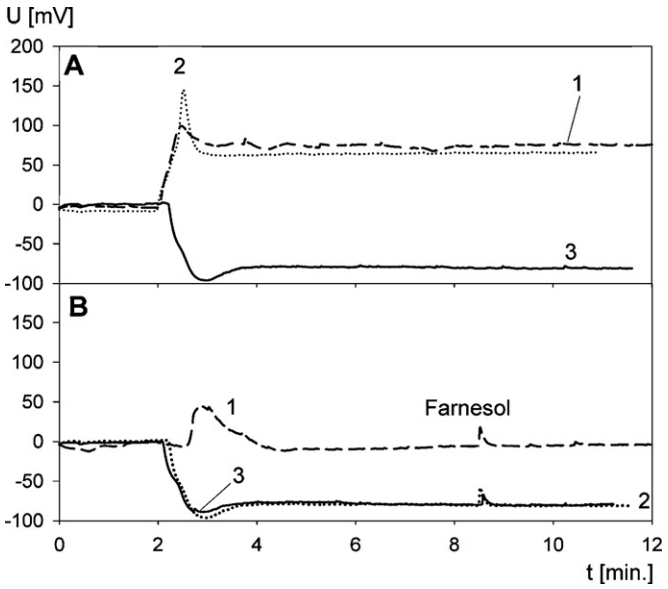


Fig. 3. CZE of medium (A) and farnesol after 24 h of growth *C. albicans* in a medium (B). Coditions, see Fig. 1; (A) medium diluted: 10^2 times (curve 1), 10^6 times (curve 2) and 10^9 times (curve 3); (B) 1×10^{-17} mol L^{-1} farnesol injected for 12 s without the media (curve 1); 1×10^{-17} mol L^{-1} farnesol added to a solution with medium diluted 10^9 times and injeced for 12 s (curve 2); farnesol poduced in medium by *C. albicans* and injected for 6 s (curve 3).

The separation of tonalid, vitamin A and cholesterol at the concetrations of each of them 1×10^{-16} mol L^{-1} is shown in Fig. 2B. The conditions of measurement were the same as those for estron and ergosterol except of PB-PEG concentration, in the sample as well as in BGE, which was necessary to be 7×10^{-5} mol L^{-1}. Although the relatively higher concentrations of analytes and lower concentration of PB-PEG have been used in the samples, higher migration times of the terpenes were reached when we compared the migration times obtained at the separation of estrone and ergosterol. It can be assumed that PB-PEG was adsorbed more on these two terpenes than on tonalid, vitamin A and cholesterol, and therefore the higher sensitivity of the estrone and ergosterol detection was achieved.

3.3. Farnesol as extracellular product of the biofilm formation at C. albicans

The farnesol is produced in the cultivating media, the source of the high concentrations of the salts and other components, e.g., amino acids. At the conditions used for the separation of fanesol alone, see chapter "3.1 CZE of farnesol" they are adsorbed onto the inner surface of the capillary and are causes of overlapping of the low concentration of farnesol present in the injected medium [39]. Therefore, dilution step of the media was necessary. In the experiments whose results are shown in Fig. 3A, curves 1–3, the alone medium was diluted in destilled water 10^2, 10^6 and 10^9 times and injected into the capillary as a sample and separated by CZE. The best results gave its dilution on 10^9. In the next steps, 1×10^{-17} mol L^{-1} farnesol was added into the 10^9 times diluted medium and into the buffer, both of them together with 5×10^{-5} mol L^{-1} PB-PEG and 1%(v/v) ethanol, see Fig. 3B, curves 1 and 2. They were injected into the capillary for 12 s. According to the results, the peak areas for farnesol were comparable. RSDs of migration times and peak areas for farnesol from diluted cultivating medium and for farnesol alone used as standard were comparable each other. Therefore, with such a large dilution of the media and supposed dilution of farnesol in the media, we can easily calculate the concentration of farnesol in unknown sample from the calibra-

tion curve, see the chapter "CZE of Farnesol". Finally, we measured farnesol produced by *C. albicans* after 24 h of the growth in a fresh medium, see Fig. 3B, curve 3. Measuring conditions were the same as those used for the analysis of farnesol above, the time of injection was 6 s. The concentration of farnesol in the real sample was calculated approximatelly as 2×10^{-9} mol L^{-1}.

4. Conclusions

CZE of terpenes dynamically labeled by the non-ionogenic tenside poly(ethylene glycol) pyrenebutanoate provides efficiency of the separation with quite reasonable sensitivity of the fluorometric detection that allowed to analyze attograms amounts of terpenes. The method has been successfully applied to the analysis of real samples of farnesol from growth media and it would be very useful to explore the principles of dynamic labeling of the small molecules. These capillary techniqes appear to be useful for fast detection and identification of terpenes in clinical samples and in samples from environment, e.g., estrogen and other substances of terpenes in drinking water.

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