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ABSTRACT

The new ultra-high performance liquid chromatography method with tandem mass spectrometry detection (UHPLC-MS/MS) has been optimized to allow fast, selective, and high-throughput analysis of two *Candida albicans* quorum sensing molecules (QSM), farnesol and tyrosol. The problem of the presence of the interference in the samples and system was successfully solved by careful optimization of chromatographic conditions. Charged hybrid stationary phase modified with pentafluorophenyl group and optimized gradient elution provided adequate separation selectivity and peak shapes. The impurity was identified as dibutyl phthalate and had the same m/zions as farnesol leading to an important interference on selected reaction monitoring channel. Two different types of biological matrices originating from vaginal fluid, supernatant and sediment, were analysed. Micro-solid phase extraction in pipette tips was optimized for the selective isolation of QSM from the supernatant. The insufficient retention of farnesol on the extraction sorbent was improved when 1% of organic solvent was added prior to extraction, while the retention of tyrosol was only possible when using combined C8 and polymer sorbent type. Strong retention of farnesol had to be solved by increasing elution solvent strength and volume up to 600 µL. However, this approach did not allow the pretreatment of sediment samples due to the sorbent clogging. Therefore, our previously developed protein precipitation method was modified and validated to analyse the sediments. New developed UHPLC-MS/MS method provided suitable accuracy and precision for the determination of QSM in vaginal fluid while using only 50 µL sample volume and two different sample preparation methods.

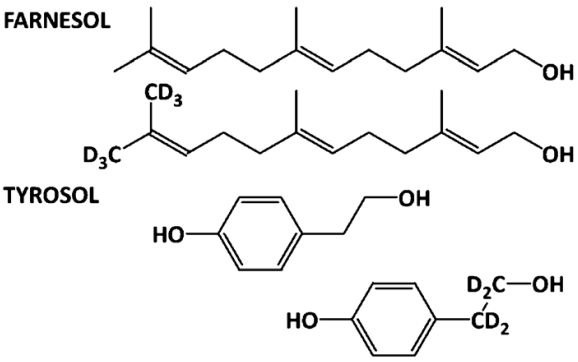
KEYWORDS:

Farnesol; Tyrosol; Quorum sensing; *Candida albicans*; Microextraction; UHPLC-MS/MS.

INTRODUCTION

Candida albicans is a common part of the healthy microbiome of the human body, particularly found on mucous membranes of gastrointestinal tract, in oral cavity, and vagina [1]. Due to the opportunistic nature, this yeast is also the most frequent cause of fungal infections in humans, especially in immunocompromised patients [2, 3]. Candidiasis manifests a wide range of clinical forms from mild skin and mucosal affections such as oral and genital thrush to life-threatening bloodstream and systemic infections [1–4]. *Candida* infections account for relevant mortality especially in tertiary health-care hospitals due to both growing population of immunosuppressed and otherwise severely ill patients and due to changing spectrum over the last decades in favour of more resistant non-*albicans* species such as *C. glabrata* and *C. auris* [2, 5, 6]. Nevertheless, *C. albicans* remains a predominant species in the vagina. About 50% of women is colonized with the yeast with the maximum load during their pregnancy [7, 8].

C. albicans is considered a part of vaginal microbiota. However, disturbance in the equilibrium with immune system or change in composition of the microbiota can lead to the development of infection. Vulvovaginal candidiasis (VVC) caused preferentially by *C. albicans* affects more than half of the female population with 5 to 9% of patients suffering from recurrent VVC [5, 8, 9]. Pathogenesis of VVC is associated with morphological transition of *C. albicans* from yeast to pseudomycelial or mycelial form depending on the growth conditions [2, 3]. The yeast-to-hyphae conversion can be induced by a wide range of chemical signals and environmental factors such as temperature, pH, CO2, glucose level, and quorum sensing molecules (QSM) which concentrations depend on fungal cell density [3, 10–12]. Several compounds with QS activity have been identified in *C. albicans*. Most attention has been paid to farnesol and tyrosol [13–15]. Farnesol supports growth of budding unicellular cells by blocking the morphological shift from commensal yeast to invasive hyphae or pseudohyphae, while tyrosol is associated with the development of pathogenic pseudo/mycelial forms [14, 16, 17]. When compared, farnesol is QSM more important and explored than tyrosol and its effect prevails [3, 12]. Farnesol is a C15 sesquiterpenic molecule with high lipophilicity. Tyrosol is a tyrosine phenolic compound with higher polarity [18]. Structures of these molecules are shown in Fig. 1. Due to the different physicochemical properties, it is rather challenging to determine both QSM in a single fast analytical run. Thus, the determinations of farnesol and tyrosol are usually carried out separately using two different methods.



summary formula	mass	Log P	pKa
$C_{15}H_{26}O$	222.20	4.828 ± 0.309	14.69 ± 0.10
$C_{15}H_{20}D_6O$	228.24	4.01	14.587
$C_8H_{10}O_2$	138.07	0.851 ± 0.204	10.17 ± 0.13
$C_8H_6D_4O_2$	142.09	1.35	9.794

Fig. 1. Structures and physicochemical properties of farnesol and tyrosol and their stable isotopically labeled internal standards [18]

For example, methods based on liquid chromatography (LC) with diode array detection (DAD) [19] or with mass spectrometry detection [20] and gas chromatography (GC) [21–26] usually with mass spectrometry (MS) detection after previous derivatization were developed for the quantification of farnesol in various matrices. A few LC methods with DAD, fluorescence, and MS detection [14, 27–29] as well as GC-MS method [30] have also been developed to determine tyrosol. All analytical methods for the determination of both QSM usually require more than 10 min with some lasting up to 45 min not accounting for time-consuming methods used for the clean-up step. In addition, they do not allow the extraction of both compounds at the same time in different types of samples. Moreover, all current methods suffer from reduced selectivity, time-consuming extraction, and in the case of GC analysis, time-consuming derivatization [20, 26, 31]. Only one GC-MS method enabling the quantification of farnesol and tyrosol in 24.33 min was published. Supernatants from fungal cultures were extracted by ethyl acetate to isolate both farnesol and tyrosol [32]. We reported in our previous study the only LC-MS method enabling the determination of farnesol and tyrosol in vaginal washing samples [33]. This method included ultra-high performance liquid chromatography (UHPLC) method with the simple isocratic elution lasting for 9 min, using column packed with C18 stationary phase, and tandem mass spectrometry (MS/MS). Protein precipitation (PP) was carried out for the sample preparation using 400 µL acetonitrile and 100 µL sample. The supernatant was directly injected to LCMS system. This work aims at the development of selective and high-throughput UHPLC-MS/MS method for the simultaneous determination of farnesol and tyrosol in small volumes of vaginal fluid. Our new method includes sample pretreatment based on miniaturized solid phase extraction. The selectivity and sensitivity of the only previously reported method [33] imperatively needed to be improved to meet more strict criteria of current bioanalysis. Therefore, our method optimization focused on (1) chromatographic separation of isobaric

interference (provided the use of unit resolution triple quadrupole), (2) use of gradient elution to avoid late elution of interferences, i.e. overcoming the important drawback of the previous methods, and increasing separation efficiency, (3) transfer to more sensitive MS platform to achieve lower LLOQs even for biological samples, (4) use of two different selected reaction monitoring (SRM) transitions to improve method selectivity, (5) application of isotopically labelled internal standards to achieve correct quantitation and to prevent matrix effects, and (6) optimization of the sample preparation methods. Our presented method now enables the exploring of the clinical importance of tyrosol and farnesol detected in vaginal swabs from women with vaginal discomfort. These compounds are usually analysed in human plasma. Although their impact and concentration in current samples can be different from those in plasma, they have never been properly examined so far.

MATERIALS AND METHODS

Chemicals and reagents

Methanol (MeOH, < 99.9%), acetonitrile (ACN, < 99.9%), formic acid, and ammonium hydroxide (25%), all of them LC-MS grade, were provided by Sigma-Aldrich (Prague, Czech Republic). Ultrapure water was produced by Milli-Q reverse osmosis system (Millipore, Bedford, MA, USA) immediately prior to use. Reference standards of farnesol (purity 96%) and tyrosol (purity 98%) were purchased from Sigma-Aldrich (Prague, Czech Republic). Deuterium-labelled farnesol (farnesol-d6; purity 99%) and tyrosol (tyrosol-d4; purity 99%), as stable isotopically labelled internal standards (SIL-IS), were obtained from TLC Pharmaceutical Standards (Ontario, Canada).

Standard solutions

The stock standard solutions of quorum sensing molecules were prepared by dissolution of each compound in ACN to solution at a concentration of 10 mg/mL for the reference standards and 1 mg/mL for SIL-IS, respectively. These solutions were stored at − 20 °C and prepared fresh every month. The diluent mixture, 40% aqueous ACN, was used for the subsequent dilution of standard solutions.

Analytical conditions

All experiments were carried out using Acquity Ultra Performance LC™ (UPLC) system (Waters, Milford, MA, USA) coupled with Xevo TQ-XS benchtop triple quadrupole mass spectrometer (Waters, Manchester, UK). The following analytical columns were tested during the optimization of separation: Acquity Ethylene Bridged

Hybrid (BEH) phenyl (100 × 2.1 mm, 1.7 µm), Acquity BEH Shield RP18 (100 × 2.1 mm, 1.7 µm), Acquity charged surface hybrid (CSH) Penta Fluoro Phenyl (100 × 2.1 mm, 1.7 µm), Acquity CSH PhenylHexyl (100 × 2.1 mm, 1.7 µm) all from Waters (Millford, USA), Ascentis Express pentafluorophenyl (100 × 3.0 mm, 2.7 µm) from Supelco (Bellefonte, PA, USA) and Luna biphenyl (100 × 2.1 mm, 1.7 µm) from Phenomenex (Torrance, CA, USA). Acquity CSH Penta Fluoro Phenyl (PFP) column was finally used for analyses. The analytes were separated using gradient elution with 0.075% aqueous formic acid (eluent A) and 0.075% formic acid in ACN (eluent B) at a flow rate of 0.4 mL/min. The gradient started at 10% eluent B in A and increased to 44% over 1.0 min. Then, a shallow ramp to 45% B during 2.0 min was applied and followed by a steep increase to 98% in 1.5 min that was kept for 30 s. In 4.1 min, the percentage of eluent B decreased to the original condition (10%). The injection volume was 5 µL. The total time of chromatographic separation including column equilibration was 6 min. Triple quadrupole MS/MS with electrospray ionization in positive mode (ESI+) was used for the detection of QSM. Nitrogen was employed as the desolvation and cone gas and argon as the collision gas. The ion source and ion optics parameters were optimized and finally set as follows: capillary voltage 1.0 kV, ion source temperature 150 °C, desolvation gas flow 1200 L/h, desolvation temperature 650 °C, cone gas flow 150 L/h, nebuliser gas 6.0 bar, and collision gas flow 0.15 mL/min. Cone voltages and collision energies shown in Table 1 were tuned individually for each SRM transition that was used for the compound quantification. Two specific SRM transitions, qualifier and quantifier, were optimized for each compound. SIL-IS were used for each compound to increase the selectivity in analysis of biological matrix. The MassLynx 4.2 Data System enabled MS control and data acquisition. TargetLynx was used for peak integration and data processing.

Table 1. SRM transitions for farnesol, tyrosol, and internal standards

	Tyrosol	***Tyrosol-d_4***	**Farnesol**	***Farnesol-d_6***
Retention time, min	1.22	*1.22*	3.49	*3.49*
Precursor	121.1	*125.0*	205.1	*211.2*
Precursor type	$[M\text{-}H_2O + H]^+$	$[M\text{-}H_2O + H]^+$	$[M\text{-}H_2O + H]^+$	$[M\text{-}H_2O + H]^+$
Product ion 1 (CV^a, CE^b)	103.2 (30; 15)	*106.2 (30; 15)*	121.0 (30; 15)	*121.2 (30; 15)*
Product ion 2 (CV^a, CE^b)	77.03 (30; 15)	–	108.98 (30; 15)	–
Ion ratio	1.67	–	2.46	–

[a]Cone voltage, V

[b]Collision energy, eV

Biological samples

The biological samples were obtained from women in the age from 18 to 50 years with chronic vulvovaginal complaints who participated in the study at the Department of Obstetrics and Gynecology, University Hospital Hradec Králové, Czech Republic. Pregnant women and those suffering from medical issues such as immunosuppressive therapy, cancer, and uncontrolled diabetes mellitus were not included in this study. All women in this study provided their written informed consent. The study design was approved by the Ethics Committee of Faculty of Medicine, University Hospital (July 17, 2014; no. 201408 S35). Vaginal fluid was collected using Dacron polyester swabs that were placed into the posterior fornix of the vagina for 20 s to achieve optimal saturation. After sample collection, Dacron polyester swabs were inserted in polypropylene tubes containing 1.5 mL of sterile phosphate-buffered saline pH 7.0 (PBS). The tubes were shaken for 20 min followed by centrifugation at 300×g and a room temperature for 15 min. The supernatants, extract of vaginal fluid, and sediments, which comprised epithelial cells and cell debris, were aliquoted and stored at − 80 °C until further analyses.

Sample preparation

Micro-solid phase extraction in pipette tips (μ-SPE-PT) was optimized to isolate the analytes from supernatant samples. The PP described in our previous study [33] was modified for the pretreatment of both sediment and supernatant.

Micro-solid phase extraction in pipette tips

A sample volume of 100 µL in PBS enriched with 1% ACN and 20 ng/mL of SIL-IS was loaded on the sorbent placed in pipette tip. The sorbent comprised 3 layers of poly(styrene-codivinyl benzene) (SDB) (Empore ™ SDB-RPS) and 3 layers of C8 (Empore ™ Octyl C8) 47 mm extraction disks from Supelco, Sigma-Aldrich. The µ-SPE-PT for single-use only was prepared according the previously published procedure [34, 35]. The µ-SPE-PT was placed in a microcentrifugation tube through the lid and centrifuged at specific temperature, speed, and time after each solvent application. First, the pipette tip cartridge was activated with 300 µL ACN (2200×g, 5 min, 4 °C) and 300 µL 1% ACN (2200×g, 5 min, 4 °C). After the loading step (2200×g, 15 min, 4 °C), the cartridge was washed with 100 µL water (2200×g, 10 min, 4 °C) and 100 µL 1% MeOH (2200×g, 15 min, 4 °C). The analytes were eluted using 2 × 300 µL ACN (2200×g, 5 min, 4 °C). The complete procedure is summarized in Electronic Supplementary Material (ESM), Table S1. The sample was evaporated to dryness using vacuum concentrator at room temperature and then reconstituted in 100 µL 40% ACN. Protein precipitation A sample volume of 50 µL in PBS with 20 ng/mL of SIL-IS was precipitated by ACN in a sample ratio of 4:1 (v/v). The mixture was well shaken and incubated for 10 min. After centrifugation (6953×g, 5 min, 4 °C), the supernatant was recovered, evaporated to dryness using vacuum concentrator at room temperature, and finally reconstituted in 50 µL 40% ACN.

Method validation

Both optimized µ-SPE-PT and PP methods followed by UHPLC-MS/MS analysis were validated in terms of linearity, limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), accuracy, precision, and matrix effects following the requirements of EMA (European Medicines Agency) Guideline on bioanalytical method validation [36]. The linearity was evaluated using matrix calibration plots in the range 1–150 ng/mL and standard calibration plots in the range 0.5–100 mg/mL. Each calibration point contained 20 ng/mL of SIL-IS. LOD was determined as the detector response with the minimum signal to noise ratio S/N ~ 3, LLOQ as the lowest quantified concentration level with the minimum S/N ~ 10 providing adequate results of accuracy and precision as per regulatory guideline. ULOQ was determined as the highest quantified concentration level in linear calibration range. Method accuracy and precision were determined at five concentration levels including LLOQ, within three times of LLOQ, within five times of LLOQ, 30–50% of the calibration curve, and 75% of calibration curve, to cover the validated range. Analyte-free biological matrix spiked with reference standards and SIL-IS was pretreated using the optimized sample preparation methods to achieve individual concentration levels [36]. Matrix effects were evaluated at 2 concentration levels as the comparison of peak area ratios of analytes and SIL-IS for standard solution, and peak area ratios

of analytes and SIL-IS for matrix pretreated using developed sample preparation methods and consequently spiked with the standards and SIL-IS. Standard solutions were prepared in 40% aqueous ACN [37].

RESULTS AND DISCUSSION

UHPLC-MS/MS method development

Development of the separation method was initially based on our previously published method [33]. That method used BEH C18 column, isocratic elution with the mobile phase composed of 0.075% formic acid and ACN in a ratio of 25:75 at a flow rate of 0.2 mL/min, with detection in an older type of MS platform with reduced UHPLC compatibility. The separation required 4.5 min. However, it was necessary to extend the analysis time to 9 min when precipitated biological samples were analysed to remove all ballast matrix compounds from the analytical system. This approach enabled prevention of matrix effects related to lately eluting interferences and avoided compromising method selectivity. In our current study, UHPLC method using gradient elution was preferred to remove possible late eluting interfering compounds originating from the biological material and/or other system contaminants to prevent the matrix effects and to increase the column lifetime, to decrease the analysis time, and to achieve higher separation efficiency. BEH C18 column was used for the optimization of mobile phase composition and final tuning. The generic gradient from 5 to 95% organic component in 5 min was initially used in the method optimization in single ion monitoring (SIM) mode. [M-H2O + H]+ ions for farnesol and tyrosol were used as the most intense ions in the full scan MS spectra. Formic and acetic acid at concentrations of 0.01, 0.025, 0.05, 0.075, 0.1, and 0.5% and 1, 5, and 10 mmol/L ammonium formate pH 5.0 were tested as the aqueous component of the mobile phase affecting the ionization of analytes. Ammonium formate did not facilitate the ionization of target molecules. Increasing concentration of acetic acid from 0.01 to 0.5% did not significantly improve the ionization either. On the other hand, an increase in concentration of formic acid in the mobile phase led to an increase in response for farnesol and to a decrease for tyrosol. Therefore, 0.075% formic acid was chosen as the aqueous component of the mobile phase being a compromise enabling to obtain acceptable results for both analytes. The effect of MeOH and ACN as the organic component, both in presence and absence of 0.075% formic acid, on separation and MS response was tested. Acidified ACN produced the best ionization of both molecules. As expected, separation time was shorter with ACN in comparison with MeOH. Using these conditions, an interference with m/z of 205 was systematically detected in the MS scan spectrum of the blank. This was a critical issue, as the interference had the same retention time and m/z corresponding to [M + HH2O]+ of farnesol. Thus, MS scan and product ion spectra of the blank and standard solution were carefully examined (Fig. 2). Two important m/z were observed in the MS scan spectrum of detected impurity besides 205.0 ion,

namely 279.1 and 148.8, which are ions typical of phthalates, in this case of dibutyl phthalate [38]. These types of contaminants can be easily introduced in the system as impurities that originate from different sources including sample collection plastic tubes as well as contamination from reagents stored in plastic containers and different plastic tools used during the manipulation with the sample. Because the impurity was observed in blank containing only acetonitrile, we concluded that it originated from the system and plastic tools used during the manipulation with the solvents. Due to the perfect coelution of phthalate and farnesol and low mass resolution of triple quadrupole, the product ions of m/z 205 and 149 were also present in the product ion spectra of farnesol (Fig. 2), even though they corresponded to completely different product ions structures. Although the interference could be removed by the systematic LC-MS system washing, it was both more effective and reliable to develop a method enabling the separation of farnesol and dibutyl phthalate. Indeed, the separation and monitoring of this interference is of crucial importance because the dibutyl phthalate abundance may change within the course of the study thus resulting in the misinterpretation of the measured data.

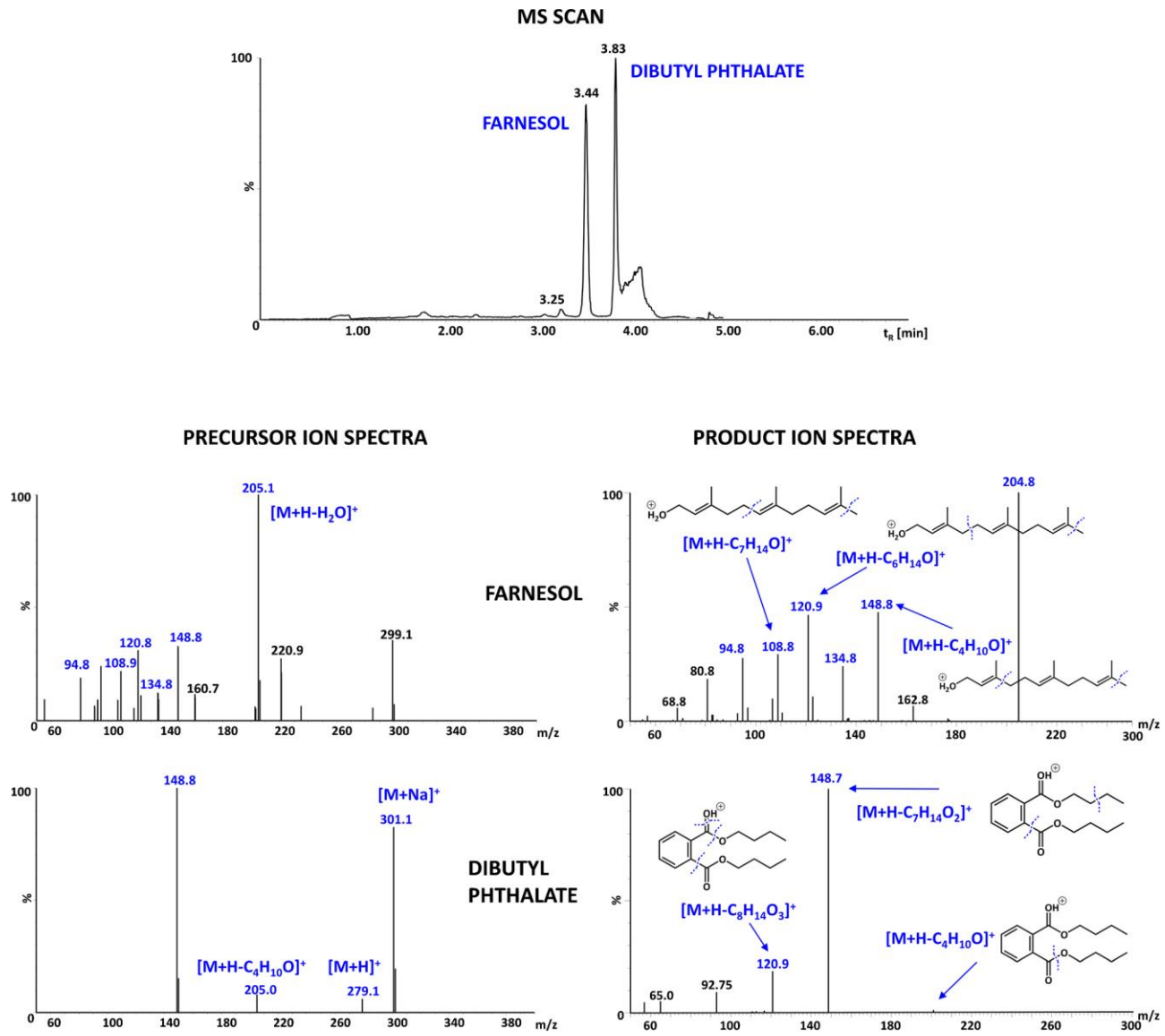


Fig. 2. MS scan and product ion spectra of farnesol and dibutyl phthalate in 40% ACN

Table 2. Comparison of chromatographic parameters obtained in method optimization from tested stationary phases. The values in bold do not meet the criteria for separation and/or selectivity

	Retention time, min		W_{50}[a], min		Resolution	Peak asymmetry	
	Farnesol	Dibutyl phthalate	Farnesol	Dibutyl phthalate		Farnesol	Dibutyl phthalate
BEH C18	**2.44**	**2.36**	0.034	0.047	**1.17**	1.04	1.47
BEH Phenyl	3.15	3.37	0.042	0.054	2.70	1.02	1.24
BEH Shield RP18	**3.05**	**2.95**	0.041	0.047	**1.34**	1.07	1.19
CSH PFP	2.43	2.63	0.036	0.063	2.38	1.05	1.51
CSH Phenyl-Hexyl	2.70	2.76	0.037	0.041	**0.91**	1.13	0.77
Ascentis Express F5	2.95	3.35	0.040	0.058	4.82	0.93	1.10
Luna Biphenyl	1.89	2.11	0.034	0.036	3.71	**1.48**	1.47

[a] Peak width at the half height

The variation in gradient profile and flow rate did not enable sufficient separation of both compounds while using analytical column BEH C18 because of the elution order and tailing of the phthalate peak which then affected also the SRM transition of farnesol. Consequently, additional analytical columns including BEH phenyl, BEH Shield RP 18, CSH Penta Fluoro Phenyl (PFP), CSH PhenylHexyl, Ascentis Express pentafluorophenyl (F5), and Luna biphenyl were tested to vary the separation selectivity and to increase the resolution of farnesol and dibutyl phthalate (Table 2 and ESM Fig. S1). Stationary phases BEH Shield RP18 and CSH Phenyl-Hexyl were excluded from the next optimization because the elution order of farnesol and dibutyl phthalate did not change in the case of BEH Shield RP18 column, or their resolution remained insufficient when CSH Phenyl-Hexyl was used. Then, the detailed optimization of gradient profile and flow rate was carried out with other columns packed with stationary phases containing phenyl functionalities. The best separation with respect to the resolution, peak shapes, and analysis time was obtained using CSH PFP column at a flow rate of 0.4 mL/min. The total analysis time was mere 6 min. The MS scan and product ion spectra of farnesol and dibutyl phthalate were determined again to compare the fragmentation patterns and to select the correct setting for SRM transitions. The product ion spectra presented in Fig. 2 show that fragmentation of farnesol and dibutyl phthalate result in two product ions with the same m/z, namely 149 and 121 presenting also the most intensive fragments of farnesol. Based on these results, two SRM transitions were used for further experiments as summarized in Table 1. The first transition of 205.1 → 121.0 was selected for quantification. Indeed, it provided high sensitivity despite being the second intensive

product ion in the fragmentation spectrum. The second transition of 205.1 → 108.8 was selected as a qualifier transition to increase the selectivity. This transition was not observed for phthalate. SRM transition of 205.1 → 148.8 was finally not used since it showed a very high intensity for the phthalate peak. Figure 3 presents the chromatograms of three most intense SRM transitions for farnesol and corresponding SRM transitions for phthalate. During all measurements, the SRM transitions for phthalate were monitored to control their separation and to avoid data misinterpretation. In contrast, optimization of SRM transitions for quantification of tyrosol was straightforward. Two SRM transitions were also monitored to increase the selectivity and sensitivity (Fig. 3).

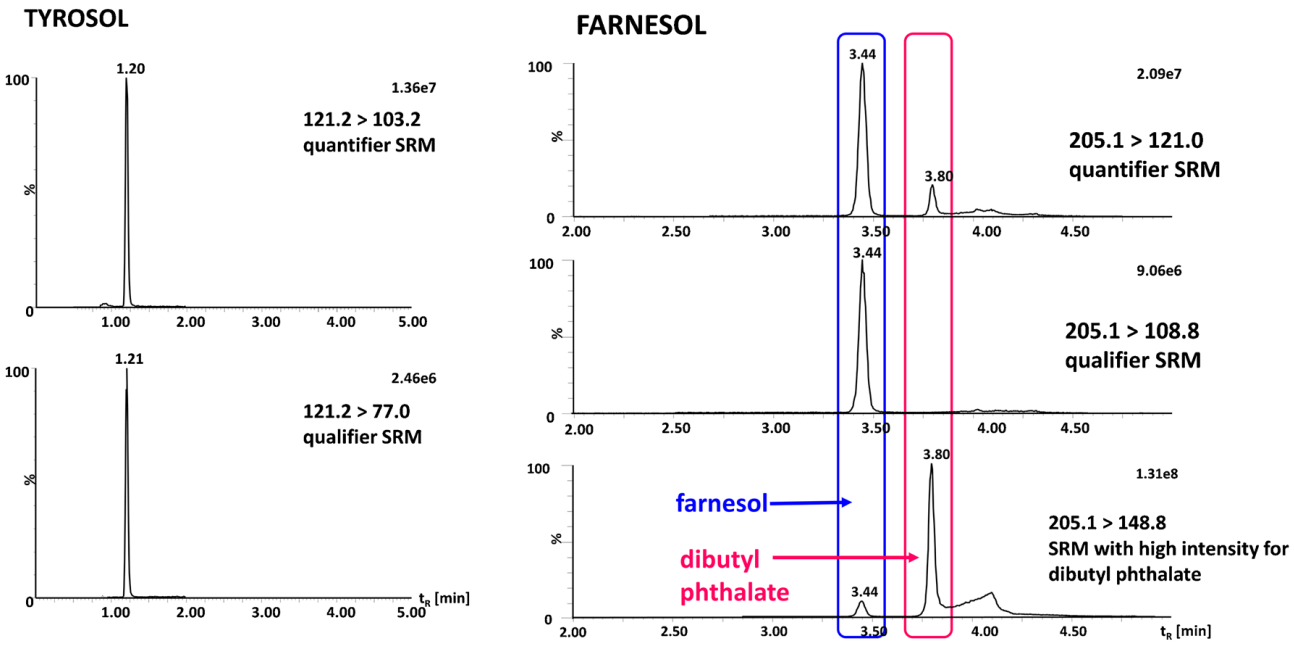


Fig. 3. The SRM chromatograms of farnesol, tyrosol, and detected impurity dibutyl phthalate

Stability study

Stability of both analytes was evaluated in PBS as the sample medium and in 40% ACN as the injection solvent under various storage conditions including storage in plastic tubes and glass vials at 20 °C considered as laboratory temperature, refrigerator at 8 °C, and deep freezer at − 80 °C. The 24-h short-term and 3-month long-term stability studies were carried out. The stability of stock solutions in ACN stored in glass vials in freezer was evaluated for 1 month. We did not observe any significant decrease exceeding 15% and the stock solution was considered stable. These studies further revealed that both analytes were stable in 40% ACN at any storage conditions in glass vials as shown in Fig. 4a. Figure 4b demonstrates some decrease in

concentration of both analytes in plastic tubes after 3-month storage in freezer while both analytes were found to be stable at laboratory temperature and in refrigerator. When the solution was stored in plastic tubes at − 80 °C, the concentration decreased to 84% for tyrosol and to 75% for farnesol, respectively. The behaviour in PBS medium was completely different pointing out on instability of farnesol. Indeed, a very poor stability of farnesol was observed particularly in those implementations when the solution was stored in plastic tubes. As shown in Fig. 4d, the final amount of farnesol was in the range 6–70% of initial concentration after 3 months. Thus, farnesol was stable only when stored in glass vials at 8 and − 80 °C with 8 and 6% of total losses, respectively. Figure 4c illustrates that the total loss of farnesol was 19% at 20 °C. Tyrosol was stable in PBS medium under all conditions (Fig. 4c, d). Based on these results, the storage in plastic tubes should be avoided when sample is stored in a buffer. In particular, non-polar farnesol is likely adsorbed at the surface of polypropylene plastic tubes used in this study. Thus, glass vials were always used for the storage of the samples containing farnesol and tyrosol in PBS medium at − 80 °C. Samples dissolved in 40% aqueous ACN can be stored both in plastic tubes and glass vials since the organic solvent prevents adsorption of farnesol.

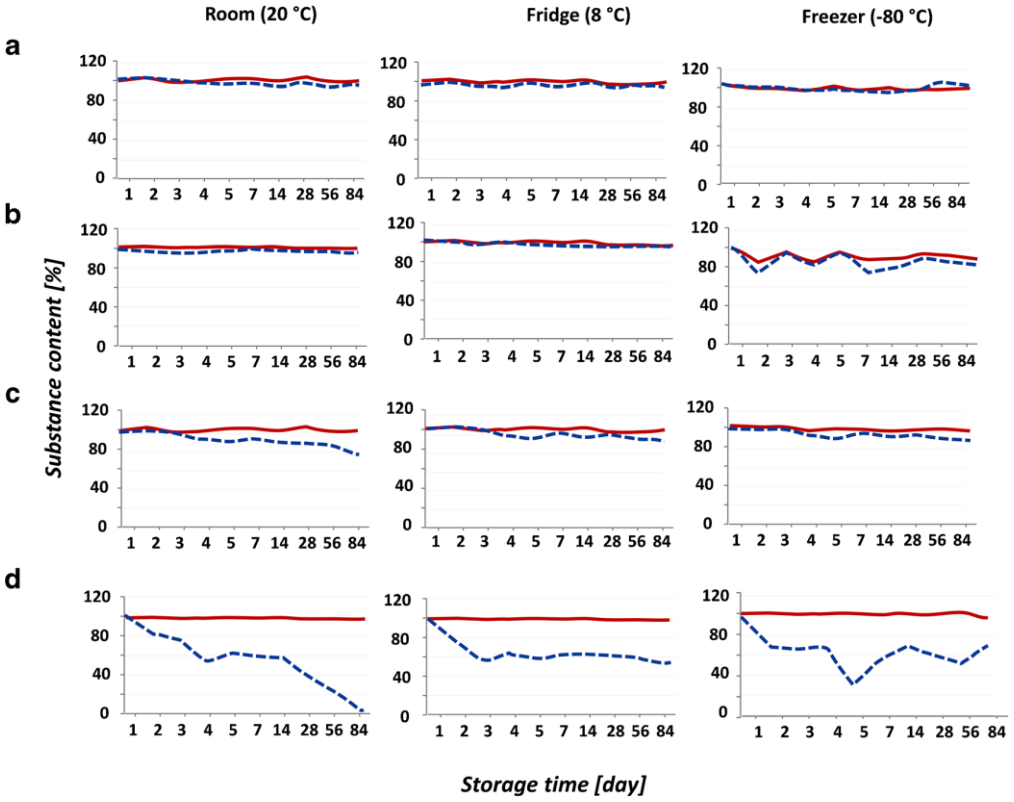


Fig. 4. Long-term 3-month stability of farnesol (blue dashed line) and tyrosol (red solid line) stored in different containers at 20, 8, and − 80 °C. Stability was tested in a 40% ACN in glass vials; b 40% ACN in plastic tubes; c PBS in glass vials; d PBS in plastic tubes

Optimization of sample preparation step The vaginal swabs were centrifuged and divided in two parts, supernatant and sediment containing the epithelial cells and cell debris. μ-SPE-PT was optimized to enable fast and selective extraction of tens of samples in short extraction time. However, the μ-SPE-PT could be used only for supernatant with low viscosity. The sediment samples clogged the sorbent during the loading step. Therefore, this technique could not be used for their extraction. This is why we had to switch to modified PP that occurred essential for success with the sediments. In addition, the supernatants were treated using the same precipitation procedure and the results of separation and quantitation were compared with those obtained using μ-SPE-PT.

Micro-solid phase extraction in pipette tips

The commercially available sorbents C8 and C18 and home-made pipette tips containing C8, C18, and SDB sorbents and combination of C8 + SDB and C18 + SDB were tested. Insufficient retention of both analytes was observed when only single sorbent was used. The recoveries for C8 and C18 were in a range of 5–43%, and 48–75% for SDB. The recoveries for farnesol and tyrosol were close to 80% when using a combination of SDB with C8 or C18. The effect of sorbent quantity in our home-made pipette tips was evaluated after filling the tips with 1 to 6 layers of SDB sorbent, and 1 to 6 layers of C8 or C18 sorbents. An increase in sorbent quantity from 1 to 3 layers led to an increase in analytes recovery due to the increasing loading capacity. No further increase in recovery was observed when 4 to 6 sorbent layers were used. A combination of 3 layers of C8 of and 3 layers of SDB was selected as the best combination enabling the retention of farnesol and tyrosol with a sufficient recovery of 71–78% and a repeatability of RSD < 7%, n = 6. Due to the potential farnesol binding to plastic material, an addition of 1–5% ACN and MeOH to the sample was tested to see the effect on the recovery by eliminating the farnesol adherence. Addition of 1% ACN led to an increase in recoveries and was included in the final protocol. ACN at percentages of 50–100% was evaluated as the possible elution agent of the compounds from the tips. The highest percentage produced the best recoveries of 73–75%. An increase in volume of ACN from 100 to 600 μL corresponding to two volumes of the pipette tip further increased the recoveries to 80–86%. The latter volume was then used in the final procedure. Finally, use of a variety of the washing solvents effectively removing ballast and interfering compounds including 0.1, 0.5, and 1% formic acid, 1, 2, and 5% ACN and MeOH, pure water, and 10 mmol/L ammonium formate at pH 5 and 8.5 were tested. We found that application of 1% MeOH, water, and ammonium formate at pH 5 minimized the losses. Also, the volume of these solvents affected the recoveries. A decrease in recovery of both analytes was a consequence of an increase in the volume from 100 to 300 μL. The two best washing

solvents providing the lowest loss of analytes were 100 µL water and 1% MeOH. They were then used in the final protocol.

Method validation

The linearity of standard calibration plot in a range of 0.5– 150 ng/mL expressed as correlation coefficient (r^2) was 0.9997 for farnesol, and 0.9993 for tyrosol (Table 3). The LLOQ was at a concentration level of 0.5 ng/mL for both compounds and the LOD corresponded to 0.16 ng/mL. The linearity for µ-SPE-PT method was determined in a range of 2–150 ng/mL and 5–150 ng/mL for tyrosol and farnesol, respectively. PP method produced the linearity in a range of 1–150 ng/mL for both analytes. This value represents a significant improvement compared with the previous state of the art since LOQ for both analytes reported elsewhere were only at µg/mL levels [19, 20, 29]. Two reports disclosed LOQ close to those determined in our current µ-SPE-PT method [28, 30]. However, when using PP in sample preparation, our method is more sensitive. The back calculated concentrations of the calibration standards were also considered. All determined values of accuracy were within the range of 92.7–103.8%, which corresponds to ± 15% of the nominal value, except for the LLOQ, which was within the range 94.4–113.8 thus being well within ± 20% as requested by the EMA regulatory guideline. The precision in this case allowed achieving RSD < 7.8% at all individual calibration levels, and RSD < 10.3% at LLOQ, which is again well within the limits of acceptance. All methods provided suitable accuracy and precision within the limits of acceptance required by EMA [36]. These parameters were evaluated at 5 concentration levels and are summarized in Table 3. The accuracy was in the range 101–116% for L1 (LLOQ) and 85–113% for L2, L3, L4, and L5, for all methods. To evaluate method precision, both methods always featured results better than 11% RSD for L1 and 7% RSD for other concentration levels. These values were significantly better than the requirement of EMA that is ≤20% for LLOQ and ≤ 15% for other concentration levels [36]. Matrix effects for all methods were evaluated using SIL-IS at LLOQ concentration level and high concentration level corresponding to 50 ng/mL. Both approaches produced fully acceptable results for both compounds. The matrix effects were determined in the range 92–106% (Table 3).

Table 3. Summary of validation results for both sample preparation techniques: microsolid phase extraction in pipette tips and protein precipitation

		Micro-solid phase extraction in pipette tips		Protein precipitation	
		Farnesol	**Tyrosol**	**Farnesol**	**Tyrosol**
Standard calibration curve, r^2		0.9997	0.9993	0.9997	0.9993
Matrix calibration curve, r^2		0.9992	0.9993	0.9997	0.9998
Concentration levels, ng/mL	*L1* (*LLOQ*)	5	2	1	1
	L2	10	5	2	2
	L3	20	10	5	5
	L4	50	50	50	50
	L5	100	100	100	100
Accuracy, %	*L1* (*LLOQ*)	101.8	103.1	103.4	116.0
	L2	93.1	112.1	101.2	112.7
	L3	95.2	111.0	85.1	97.0
	L4	93.3	110.8	88.6	103.2
	L5	92.3	109.2	96.2	94.6
Precision, RSD %	*L1* (*LLOQ*)	11.0	7.0	5.9	6.0
	L2	3.3	1.5	3.8	6.0
	L3	2.4	3.3	5.8	6.7
	L4	0.8	2.5	3.2	3.0
	L5	1.6	1.8	3.6	1.9
Matrix effects, %	*L1*	93.6	92.6	93.3	104.3
	L4	105.6	102.4	99.4	96.3

Analysis of biological samples using developed UHPLC-MS/MS method

PP and µ-SPE-PT were applied to the biological samples obtained from vaginal swabs. Quantification of farnesol and tyrosol in vaginal fluid was carried out using the matrix calibration plot containing SIL-IS at the

concentration range relevant to the biological samples. Both 17 supernatant and 14 sediment samples collected from 17 women were evaluated for presence of farnesol and tyrosol. All measured sediments and supernatants contained tyrosol. Detected and quantified concentrations were in the range of 4–100 ng/mL. Farnesol was not detected in supernatants, while low concentrations were found in several sediments. Only in one case, the concentration of farnesol was higher than 10 ng/mL as presented in Table 4. The concentration levels in supernatants for farnesol and tyrosol pretreated by PP and µ-SPE-PT were compared using t test pairwise comparison at the significance level α = 0.05. This t test showed that the results were identical on the significance level α = 0.025 as shown in Fig. 5a. The chromatograms of supernatant containing only tyrosol and chromatograms of sediment containing both analytes are shown in Fig. 5b.

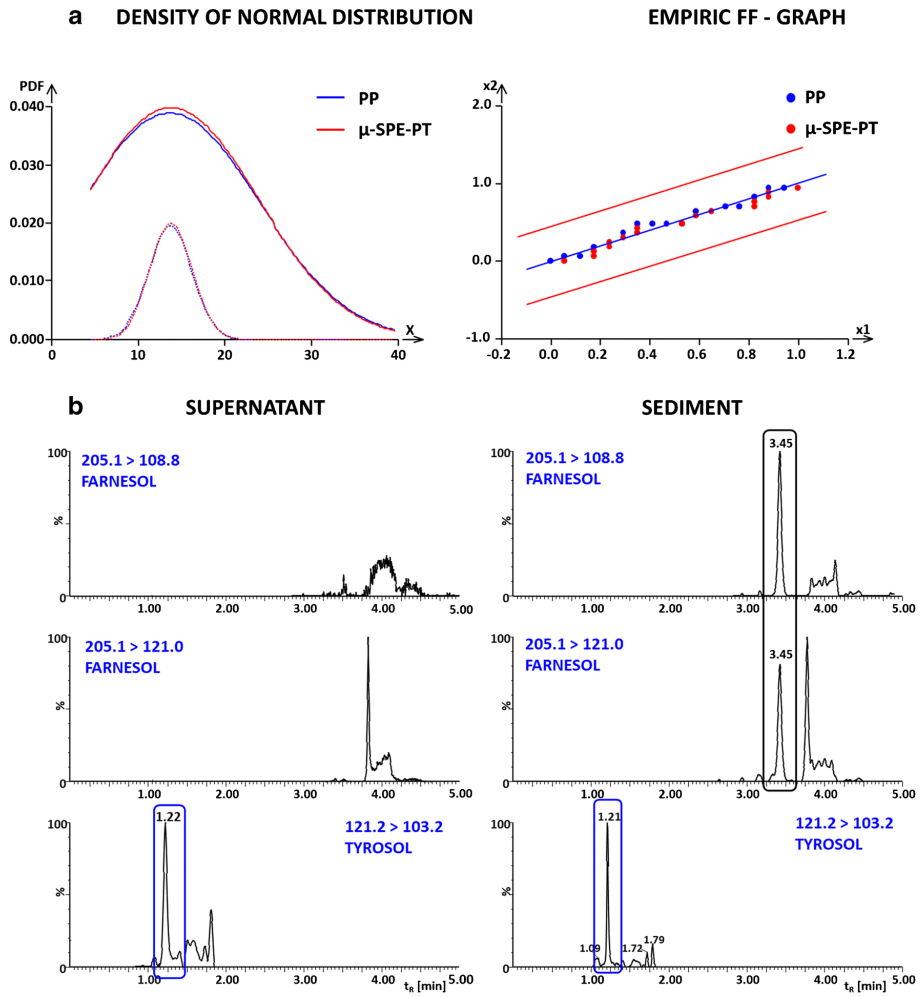


Fig. 5: The final results obtained with biological samples containing QSM: a t test pairwise comparison of the results at level of significance α = 0.05 consistency of supernatant samples pretreated by protein precipitation and µ-SPE-PT methods; b example chromatograms of sediment and supernatant obtained for sample containing farnesol and tyrosol

Table 4. The amount of quorum sensing molecules, farnesol and tyrosol, in vaginal fluid in women with vulvovaginal discomfort, x means the sample was not available for the determination, - means the compound was not detected

Sample no.	Concentration (ng/mL)					
	Protein precipitation				Micro-solid phase extraction in pipette tips	
	Sediment		Supernatant		Supernatant	
	Tyrosol	Farnesol	Tyrosol	Farnesol	Tyrosol	Farnesol
1	7.5	–	10.4	–	11.9	–
2	5.6	–	6.0	–	5.8	–
3	x	x	4.8	–	4.6	–
4	10.2	–	8.7	–	7.6	–
5	LOD	1.0	4.4	–	5.6	–
6	6.0	LOD	6.4	LOD	6.5	–
7	16.3	1.3	14.1	–	12.4	–
8	8.0	LOD	8.0	–	7.1	–
9	14.0	1.0	11.9	–	11.9	–
10	16.5	8.1	12.8	–	14.1	–
11	x	x	5.4	–	6.4	–
12	x	x	17.3	–	20.5	–
13	31.2	1.0	26.4	–	27.0	–
14	42.6	14.2	34.8	–	28.8	–
15	98.9	6.2	38.1	–	39.6	–
16	6.8	1.0	7.0	–	6.1	–
17	14.4	2.1	16.2	–	18.7	–

CONCLUSIONS

Fast, selective, and sensitive UHPLC-MS/MS method enabling the determination of two QSM, farnesol and tyrosol, has been developed. In comparison with our previous method, this new one allows more sensitive and selective analysis in a shorter time. The optimized gradient profile and carefully selected stationary phase enabled baseline separation not only of farnesol and tyrosol but also of dibutyl phthalate interference that was most of the time present in the system and could mislead the interpretations. The MS/MS detection using two SRM transitions further ensured increase in selectivity in testing of biological material. We also report for the first time the lack of stability of farnesol in PBS medium at various storage conditions. Moreover, its binding to plastic material of the Eppendorf tubes had to be eliminated during the method development. The easiest way relied on use of glass containers and low temperatures that enabled problem-free storage of analysed samples without losses of analytes. μ-SPE-PT and PP sample preparation techniques were optimized and fully validated with very good accuracy, precision, and linearity even when using small quantities of biological matrix. μ-SPE-PT was applied for the determination of QSM only in vaginal swab supernatants since the clogging of the sorbent was observed when sediment was loaded. Previously developed PP was modified and validated for the pretreatment of both types of samples. The QSM concentrations in supernatant samples determined by PP and μ-SPE-PT were close with no significant differences. Our current PP method is suitable for both types of samples, simple, fast, cheap, and easy to implement just to name some of its undoubted advantages. The PP-UHPLC-MS/MS method can be easily used for large clinical sample sets while examining women with vulvovaginal complaints. Such studies can be used to explore and explain the effect of QSM on the development of candidiasis. Our results confirm that presence of tyrosol in vaginal fluid is in accordance with preferences of hyphal or pseudohyphal form for invasion of epithelial cells of vaginal mucosa [3]. Further study will focus on the relevance using an even larger number of specimens from women with and without vaginal complaints. Moreover, the measurement of large sample sets by non-targeted UHPLC method coupled to high resolution mass spectrometry will be carried out. Optimization and validation of PP as a less selective sample preparation method providing more generic approach in nontargeted screening was thus another rationale.

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Conflict of interest

The authors declare that there is no conflict of interest.

Ethics approval

The study design was approved by the Ethics Committee of Faculty of Medicine, University Hospital (July 17, 2014; no. 201408 S35).

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