Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 674–681

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Ultra high performance liquid chromatography tandem mass spectrometry analysis of quorum-sensing molecules of *Candida albicans*

Petr Greguš[a], Hana Vlčková[a], Vladimír Buchta[b], Jan Kestřanek[c], Lucie Křivčíková[b], Lucie Nováková[a,*]

[a] Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Heyrovského 1203, 500 05 Hradec Králové, Czech Republic
[b] Department of Clinical Microbiology, Faculty of Medicine in Hradec Králové, Charles University, University Hospital, Sokolská 581, 500 05 Hradec Králové, Czech Republic
[c] Department of Obstetrics and Gynecology, Faculty of Medicine in Hradec Králové, Charles University, University Hospital, Sokolská 581, 500 05 Hradec Králové, Czech Republic

ARTICLE INFO

Article history:
Received 7 April 2010
Received in revised form 28 May 2010
Accepted 28 May 2010
Available online 8 June 2010

Keywords:
Quorum sensing
Farnesol
Tyrosol
UHPLC–MS/MS
Bio-analytical method

ABSTRACT

Candida albicans is generally one of the most commonly isolated fungal pathogen from human body. It is a frequent cause of nosocomial infections, bloodstream infections, urinary infections and mucosal infections of oral cavity and vagina *C. albicans* can grow as hyphae, pseudohyphae, or budding yeast. Morphological conversion of a yeast form to pseudohyphal or hyphal one is often characterized by the change of commensal status to an invasive form. Farnesol and tyrosol can participate in these transformation processes as quorum sensing molecules together with some physical–chemical factors.

A new analytical method for identification and quantification of biologically active substances farnesol and tyrosol using ultra high performance liquid chromatography (UHPLC) in connection with tandem mass spectrometry was developed. The analytes were separated on Acquity BEH C18 analytical column using binary mobile phase consisting of acetonitrile and formic acid 0.075% (75:25) at flow-rate 0.20 ml/min. SRM (selected reaction monitoring) mode was applied in order to ensure sufficient selectivity and sensitivity using the first most intensive transition as a quantitative (121 > 77 and 205 > 121) and second one for the confirmation purposes (121 > 93 and 205 > 109). The method was validated in terms of linearity (>0.9994), precision (0.5–3.8% RSD), accuracy (78.9–106.0%), LOD (limit of detection) and LOQ (limit of quantitation). The method can serve as an analytical tool for the detection and determination of quorum-sensing molecules in biological samples.

1. Introduction

The polymorphic fungus *Candida albicans* is one of the most important yeast in medicine. It is a member of the indigenous microbiota of mucosa and skin in humans and animals and is thought to be acquired during passage through the birth canal. *C. albicans* has been recently used as a model for studying the basic biology of fungi as well. For fungi exhibiting yeast–mycelium dimorphism the dependence of cell morphology on initial cell density has been termed an inoculum size effect. The inoculum size effect in the dimorphic yeast results from the production of quorum-sensing molecules (QSMs). The QSMs indentified in *C. albicans* are farnesol, farnesic acid, and tyrosol [1–6].

QSMs are extracellular chemical signals, and are produced continuously in response to increasing density of microbial population to coordinate action of the cells. Their production is usually not dependent on the type of carbon source or nitrogen source or on the chemical nature of the growth medium. In general, these signals can regulate some important virulent, morphological, and physiological properties through activation of proper genes. Majority of studies concern QSM of bacteria [7,8], but there is a growing number of reports about QSM in fungi, especially yeasts. The main two QSMs in *Candida* under study are farnesol and tyrosol, their structures are shown in Fig. 1. While farnesol blocks the yeast-to-mycelial dimophic transition of *C. albicans*, tyrosol supports the development of filamentous form of this yeast [5,6,9].

Originally, analytical methods for the determination of farnesol and tyrosol were developed separately. Farnesol was first discovered to be QSM of *C. albicans* [1], while tyrosol function in the QS was described later [2]. It would be however highly convenient to develop one analytical method being able of simultaneous determination of farnesol and tyrosol, because such method would give direct information about the concentrations of both QSM independently of sample origin and thus enable better identification of cell morphology and description of invasive stadium. This could be helpful in diagnostic and treatment approaches, e.g. in vaginal candidosis.

Farnesol is a volatile molecule of terpenoid structure, therefore GC–MS (gas chromatography with mass spectrometry detection) was often a method of choice for its determination [1,10,11]. Both

* Corresponding author. Tel.: +420 495067381; fax: +420 495067164.
E-mail address: nol@email.cz (L. Nováková).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.05.029

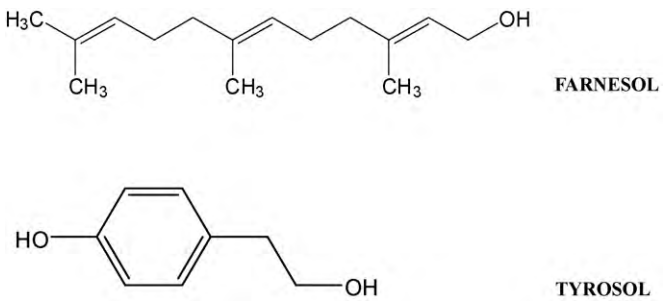

Fig. 1. Chemical structures of farnesol and tyrosol.

chemical ionization and electron ionization were employed. These methods however were developed only for qualitative purposes to confirm a presence of farnesol molecule in various matrices including *C. albicans* strains [1], *Mycastor coypus* anal scent glands [10] or tobacco smoke [11]. Further, HPLC–UV (high performance liquid chromatography with ultra-violet detection) was used for the identification purposes using retention times [1], however in complicated matrices this approach might miss sufficient selectivity. Further, farnesol metabolites (farnesyl-glucuronide) and farnesol with other related compounds were determined using HPLC in connection with ESI-MS (electrospray ionization mass spectrometry) [12,13]. Ionization was typically performed in negative ion mode. $[M-H]^-$ served as a precursor ion for further MS/MS quantitation of farnesol or its glucuronide. According to our knowledge, only one method was developed for quantitative purposes and fully validated for the determination of farnesol in rat liver and testis [12]. ESI-MS/MS detection is highly advantageous from the selectivity and sensitivity point of view. However, the procedure reports a necessary derivatization step in order to reach sufficient sensitivity, which complicates the method and makes the application time-consuming. No fast, simple and sensitive method for the qualitative and quantitative analyses of farnesol was found in the literature. An overview of analytical methods used for the determination of farnesol and its related compounds is shown in Table 1.

Tyrosol was often determined with other phenolic compounds in virgin olive oils [14,15] or in human LDL-fraction after digestion of olive oil [16,17] or in olive mill wastewaters [18]. HPLC using UV (typically 280 nm) or MS detection was a method of choice [14–22]. MS detection is unequivocally more convenient, because it provides better sensitivity and selectivity especially in complicated matrices. Most of HPLC methods were developed and fully validated for quantitative purposes using ESI-MS/MS which provides very high selectivity in complicated matrices [14,16–18]. ESI was performed in negative ion mode using $[M-H]^-$ as a precursor ion for further SRM (selected reaction monitoring) quantitation. Typical sensitivity of such methods reached LOQ (limit of quantitation) around 1 ng/ml levels. Other methods for the determination of tyrosol employed GC–MS with EI [21] or MEKC (micellar electrokinetic chromatography) [22]. An overview of analytical methods used for the determination of tyrosol and its related compounds is shown in Table 2.

The aim of this work was to develop fast, reliable, sensitive and selective analytical method for the simultaneous determination of farnesol and tyrosol as quorum-sensing molecules of *C. albicans* using UHPLC–MS/MS method. While tyrosol was quite successfully evaluated in terms of quantity using specific ESI-MS/MS methods (although not all method provided all quantitative and validation results), mostly only qualitative analysis of farnesol was typically performed. According to our knowledge, simultaneous determination of farnesol and tyrosol has never been performed before. Analytical approaches employed various instrumentations for the analysis of the two analytes. GC was often used for the analysis of

Table 1
An overview of analytical methods used for the determination of farnesol and its relative substances.

Determined substances	Matrix sample prep.	Method/stationary phase	Mobile phase	Detection	Validation data	Analysis time	Ref.
Farnesol	*Candida albicans* Extraction by ethyl acetate	**HPLC**-RP C18 5 μm 4.6 mm × 250 mm	MeOH:H_2O (4:1)	UV 210 mm	Qualitative method	NA	[1]
Farnesol	*Candida albicans* Extraction by ethyl acetate	**GC**-DB5 column 30 m	NA	EI-MS CI-MS	Qualitative method	11 min	[1]
Farnesol and isomers Esters of farnesol	*Mycastor coypus* - anal scent glands	**GC**-ZB-FFAP Coated capillary column 30 m × 0.25 mm, 0.25 μm	Helium	EI-MS CI-MS	Qualitative method	NA	[10]
	Flash chromatography	XTI-5 coated capillary col 5 m × 0.25 mm, 0.25 μm		IR			
Farnesol Farnesylacetone Farnesyl-methylether	Tobacco smoke Extraction by hexane	**GC**-capillary column (122-5562, DB-5MS 5% phenyl 95% methyl, 15 m × 0.20 mm, 0.33 μm film)	NA	EI-MS	Qualitative method	NA	[11]
Farnesol, geranyl-geraniol	Rat liver and testis LLE	**HPLC**-Symmetry Shield C8 5 μm 2.1 mm × 150 mm IS = n-pentadecanol	ACN, 10 mM ammonium acetate, acetic acid (90:10:0.1)	ESI-MS/MS $[M-H]^-$ derivatization	rec = 108–111% LOQ = 0.15 ng/g LOD = 0.05 ng/g	8 min	[12]
Farnesyl-glucuronide	Liver microsomes Sonication	**HPLC**-Spherisorb ODS 5 μm 2.1 mm × 150 mm	A: 0.1% formic acid B: ACN + 0.1% formic acid, gradient elution	ESI-MS/MS $[M-H]^-$	LOD = 30 fmol	7 min	[13]

Abbreviations: MS, mass spectrometry; ESI, electrospray ionization; EI, electron ionization; CI, chemical ionization; ACN, acetonitrile; MeOH, methanol; IS, internal standard; rec, recovery; LLE, liquid–liquid extraction; NA, data not available.

Table 2
An overview of analytical methods used for the determination of tyrosol and its relative substances.

Determined substances	Matrix sample prep.	Method/stationary phase	Mobile phase/electrolyte	Detection	Validation data	Analysis time	Ref.
Hydroxytyrosol **tyrosol**	Virgin olive oil	**HPLC**-Luna C18	A: H_2O + 0.1% HCOOH	DAD 280, 240, 320 nm	HPLC–DAD	40 min	[14]
Phenolic acids Vanillin	SPE	5 μm, 2.0 mm × 150 mm	B: ACN Gradient elution	ESI-MS/MS $[M-H]^-$	LOD = 0.28 μg/ml LOQ = 0.39 μg/ml Rec = 76–115%		
Tyrosol Hydroxytyrosol	Spanish and Italian extra virgin olive oils LLE	**HPLC**-Luna RP18 5 μm, 4.6 mm × 250 mm	A: HCOOH pH 3.2 in water B: ACN	DAD ESI-MS	NA	50 min	[15]
Tyrosol, **hydroxytyrosol**, homovanillic acid Phenolic metabolites	Human LDL-fraction SPE	**HPLC**-Luna C18 5 μm, 2.0 mm × 150 mm IS = taxifolin	A: 0.1% HCOOH B: 100%ACN Gradient elution	ESI-MS/MS $[M-H]^-$	LOD = 0.32 ng/ml LOQ = 1.08 ng/ml Rec = 69–80%	25 min	[16]
Hydroxytyrosol phenolic compounds Homovanillic acid	Human LDL-fraction SPE	**HPLC**-Luna C18 3 μm 2.0 mm × 50 mm IS = taxifolin	A: 0.1% HCOOH B: 100% ACN Gradient elution	ESI-MS/MS $[M-H]^-$	Rec = 82% LOD = 0.24 ng/ml LOQ = 0.81 ng/ml	6 min	[17]
Phenolic compounds, hydroxytyrosol, **tyrosol**, caffeic acid	Olive mill wastewaters SPE	**HPLC**-C8 XTerra 3.5 μm, 2.1 mm × 150 mm IS = 1-hydroxyphenyethanol	A: acetic acid in water B: ACN Gradient elution	UV 275 nm 230 nm ESI-MS/MS $[M-H]^-$	LOD = 2×10^{-6} g/ml LOQ = 7×10^{-6} g/ml	12 min	[18]
Tyrosol	*C. albicans* biofilm SPE	**HPLC**-Spherisorb C18 5 μm 4.6 mm × 250 mm	ACN:H_2SO_4 1 mM (10:90)	DAD	LOQ = 1 μM Rec = 92.8 ± 0.8%	10 min	[19]
Tyrosol, resveratrol	Rabbit platelet	**HPLC**-Zorbax Eclipse XDB C8 5 μm 4.6 × 150 mm	MeOH:H_2O (40:60)	UV 256 nm	NA	30 min	[20]
Acetylated derivatives	Acetylation, evaporation and reconstitution			ESI-MS $M-H]^-$			
Tyrosol Hydroxytyrosol	Human urine LLE	**Capillary GC-**HP Ultra 2 (12.5 m × 0.2 mm, 0.33 mm film)	Helium	EI-MS SIM	LOD = 0.6 ng/ml LOQ = 1.7 ng/ml	11 min	[21]
Tyrosol, tryptophol, ferulic acid	Sake SPE	**HPLC**-L-ODS 4.6 mm × 150 mm	H_2O:ACN (85:15)	UV 280 nm	NA	20 min	[22]
Tyrosol, tryptophol, ferulic acid	Sake SPE	**MEKC** Fused silica capillary	SDS in borate buffer pH 8.5	UV 280 nm	LOD 0.05 μg/ml	5 min	[22]

Abbreviations: MS, mass spectrometry; ESI, electrospray ionization; EI, electron ionization; CI, chemical ionization; ACN, acetonitrile; NA, not available; MRM, multiple reaction monitoring; MEKC, micellar electrokinetic chromatography; IS, internal standard; rec, recovery; SDS, sodium dodecyl sulphate; SIM, single ion monitoring; SPE, solid phase extraction; LLE, liquid–liquid extraction.

farnesol, while HPLC for the analysis of tyrosol. Unfortunately, most analytical methods for tyrosol were developed for its determination with many others phenolic compounds, therefore the methods were unacceptably time-consuming (10–50 min) for the routine bio-analytical purposes. The only shorter runs could have been observed using MEKC for the analysis [22] or if only hydroxytyrosol was analyzed without tyrosol [17]. Applicability of the method for the determination of farnesol and tyrosol in vaginal washings has also never been referred before. Such a method is necessary for the evaluation of correlation of concentration of QSM with clinical status of patients with vaginal candidosis. High selectivity and sensitivity was assured by MS/MS detection; UHPLC increased the speed and efficiency of the method.

2. Experimental

2.1. Chemicals and reagents

Working standards of farnesol (*trans*,*trans*-3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) and tyrosol (2-(4-hydroxyphenyl) ethanol), ≥99.5% (GC), were obtained from Sigma–Aldrich (Prague, Czech Republic).

The acetic acid, reagent grade, the formic acid, reagent grade and the acetonitrile, LC–MS grade, were purchased from Sigma–Aldrich. HPLC grade water was prepared by Milli-Q reverse osmosis Millipore (Bedford, MA, USA) and it meets European Pharmacopoeia requirements.

2.2. Chromatography

UHPLC (ultra high performance liquid chromatography) system Acquity UPLC (ACQ) (Waters, Prague, Czech Republic) was used for the purposes of this study. It consisted of ACQ-binary solvent manager, ACQ-sample manager and ACQ-tunable UV detector. All UHPLC analyses were performed on BEH C_{18} analytical column (100 mm × 2.1 mm, 1.7 μm, Waters, Prague, Czech Republic) based on Bridged Ethyl Hybrid (BEH) particles. Mobile phase was composed of acetonitrile and formic acid 0.075% (75:25) using isocratic elution. Mobile phase flow-rate was 0.20 ml/min. The analytical column was kept at 35 °C by column oven. The solutions were stored in the auto-sampler at 4 °C. The partial loop with needle overfill injection mode was set up to inject 3 μl using 5 μl injection loop. Acetonitrile was used as a strong wash and 20% acetonitrile in water was used as a weak wash solvent.

2.3. Mass spectrometry

A Quattro Micro (Micromass, Manchester, UK) triple quadrupole tandem mass spectrometer equipped with a multi-mode ionization source (ESCI) was used in this study. This ion source enables high-speed switching between electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) within one ion source.

Ion source set-up was tuned as follows: capillary voltage: 3200 V, ion source temperature: 130 °C, extractor: 3.0 V, RF lens: 1.0 V. The desolvation gas was nitrogen at flow 500 l/h and at the temperature 450 °C. Cone voltage was set up individually for each analyte (Table 3). Nitrogen was used also as a cone gas (120 l/h) to prevent the contamination of sample cone. Quantitation of all analytes was performed in ESI positive ion mode using SRM (selected reaction monitoring) experiment. Two specific transitions were optimized for each molecule in order to increase selectivity of the method. Argon was used as collision gas and collision energy was optimized for each analyte individually (Table 3).

The MassLynx 4.1 Data System was used for data MS control and data gathering. QuanLynx software was used for data processing and quantitation—regression analysis of standard curves and calculation of concentrations.

Table 3
Optimization of specific transitions for farnesol and tyrosol, SRM1 = quantitative transition, SRM2 = confirmative transition.

Compound	Tyrosol MW = 138.07		Farnesol MW = 222.20	
t_R [min]	1.24		3.85	
Precursor	121.2		205.3	
Precursor type	$[M+H-H_2O]^+$		$[M+H-H_2O]^+$	
SRM	SRM1	SRM2	SRM1	SRM2
Fragment	77.1	93.0	121.1	109.1
Dwell time	0.2	0.2	0.2	0.2
Cone voltage	25	25	20	20
Collision energy	20	10	10	10
Secondary ion ratio	6.2		2.1	

2.4. Preparation of standard solutions

The stock solutions of standards were prepared by dissolving of the amount corresponding to 10.0 mM concentration of appropriate working standard into 20.0 ml of pure acetonitrile in volumetric flask (0.0276 g for tyrosol and 0.0444 g for farnesol). Stock solutions were further diluted by acetonitrile to achieve a concentration 500 nM for SST (System suitability test) measurements, and to get individual points of calibration curve in the range 10–1000 nM, using seven calibration points (1000, 500, 200, 100, 50, 20 and 10 nM).

2.5. Sample preparation

Biological samples were prepared using deproteinization step with acetonitrile. 100 μl of vaginal washing was used for each analysis. Each sample was prepared in triplicate. Acetonitrile was added to the sample in the ratio of 4:1. The sample was well shaken and subsequently filtered via 0.20 μm PTFE filter. The filtrate was injected onto UHPLC system.

2.6. System suitability test and validation

An important part of method validation is the SST, details of which are usually given in Pharmacopoeias [23,24]. The SST was performed under optimized chromatographic conditions. In mass spectrometric methods only repeatability of retention times and peak area is checked.

Method validation was performed in accordance with ICH (International Conference on Harmonization) requirements [25]. Calibration curves of both analytes in the concentration range 10–1000 nM were measured using mixed standard solutions in acetonitrile. Lower limit of quantitation and limit of detection were established based on signal-to-noise (S/N) ratio approach. Limit of detection was expressed as S/N = 3, lower limit of quantitation was expressed as S/N = 10. Method precision and method recovery were established using deproteinization procedure as an inherent part of the method. For the precision, biological samples (vaginal washings) at three different concentration levels, in the range of calibration curve, were measured in three replicates to calculate RSD, which describes the closeness of agreement between series of measurements. Method accuracy was determined using spiked vaginal washing samples treated by sample preparation procedure (standard addition method was used, because no blank samples are available and real samples might contain either farnesol and/or tyrosol), again at three different levels in three replicates in order to establish the closeness of agreement between the true and measured value as it corresponds to ICH requirements [25].

Selectivity and matrix effects were verified using biological samples without the content of tyrosol or farnesol as follows: for the determination of selectivity the injection of vaginal washing treated by the same sample preparation step was used. Matrix effects were established using direct inlet by Hamilton syringe, where mixed standard solution was introduced to the mass spectrometer by direct infusion and the vaginal washing was injected by the auto-sampler to observe matrix suppressions or enhancements as positive or negative peaks influencing data plot of analytes.

2.7. Biological samples

Vaginal washing samples were used for the determination of QSM farnesol and tyrosol in biological samples. These samples were obtained from women with and without vulvovaginal discomfort. In order to investigate local parameters in vagina a small resterilizable probe was designed. Vaginal washings were gained with the help of a special spoon-shaped chamber. The chamber was equipped with valve for a syringe with sterile water. The point of these chambers was a possibility to determine precisely the area of the washed mucosa. The amount of the liquid was also precisely set.

The chamber was closely placed to anterior vaginal wall. Liquid was injected into vagina and sucked up afterwards. The taken liquid was divided in 1 ml into test tubes. The samples were processed in microbiological laboratory to isolate and identify yeasts if present. One part of sample was frozen at −80 °C for analytical purposes.

3. Results and discussion

3.1. Ultra high performance liquid chromatography and mass spectrometry

The advantage of ESCI ionization source was benefited and all ionization approaches ESI +/− and APCI +/− were examined in order to get the best response for both analytes. As farnesol and tyrosol are small molecules of not substantial polarity, both ionization modes could be expected as convenient. Majority of published works employed ESI (see Tables 1 and 2), however, both positive and negative ionization modes were applied. In full-scan spectra protonated molecule $[M+H]^+$ was not observed neither in electrospray nor in atmospheric pressure chemical ionization (APCI) record. The molecule was directly fragmented in the ion source even under mild ionization conditions, thus both analytes farnesol and tyrosol provided a precursor ion $[M+H-H_2O]^+$ in positive ion mode, while in negative ion mode $[M-H]^-$ was observed. The best response and S/N ratio was obtained in ESI positive ion mode, thus precursor ion $[M+H-H_2O]^+$ was further used for quantitation and fine tuning of all parameters of mass spectrometer (see Section 2.3). Cone voltage was set up individually for each analyte—the results could be seen in Table 3.

UHPLC was used as separation method for the analysis of farnesol and tyrosol under isocratic conditions. Acquity BEH C18 was chosen for the separation with the regard to physical–chemical properties of the analytes and bibliographic information (Tables 1 and 2). Minimally 70% of acetonitrile need to be used in order to obtain good resolution between the peak of farnesol and tyrosol. The separation of analytes was developed with the regard to mass spectrometric detection, which limits applicability of solvents. Only few additives are volatile enabling sensitive mass spectrometric response. Formic acid and acetic acid at different concentrations were tested in this study (Fig. 2). In compromise of the response of mass spectrometer, analysis time and resolution, acetonitrile in combination with acetic acid 0.075% was finally chosen as mobile phase additive in the ratio 75:25. The flow-rate was 0.2 ml/min in accordance with the requirements of electrospray ionization.

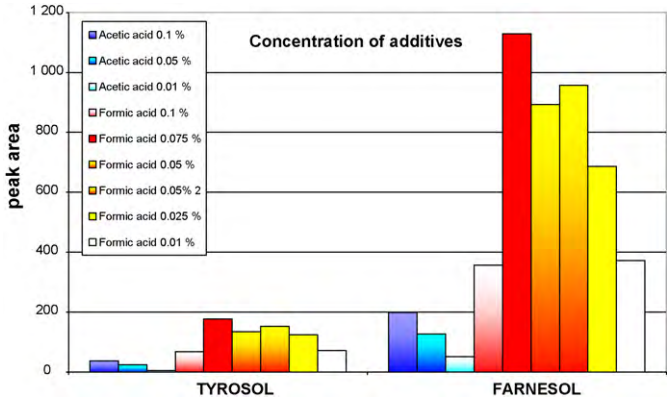

Fig. 2. Optimization of mobile phase additives—the influence of formic acid and acetic acid on ionization of analytes.

Quantitation of both analytes was performed in ESI positive ion mode using SRM (selected reaction monitoring) mode. Two specific transitions (quantitative SRM1 and confirmatory SRM2) were optimized for both molecules in order to increase selectivity and identification value of the method. For this reason also the ratio of SRM1 and SRM2 was calculated (secondary ion ratio—see Table 3). Product ions were chosen according to the fragmentation pathways in Product ion scan mode (see Fig. 3). Collision energy was optimized for each analyte and for each of its two transitions individually in order to obtain the highest sensitivity (see Table 3).

3.2. Sample preparation

Deproteinization was chosen as sample preparation step as it is fast, simple and effective. The convenience of developed sample preparation for the purposes of analysis of vaginal washings was proven by validation. Acetonitrile was used as deproteinization agent followed by filtration through fine 0.2 μm PTFE filters.

3.3. System suitability test and validation

The SST was performed by 10 subsequent injections of standard mixture of farnesol and tyrosol at the concentration 500 nM. The repeatability of retention times and peak areas was checked and it was expressed as RSD in %. Excellent repeatability of injection was obtained for both retention time (RSD < 1%) and for peak area (RSD < 4%) (see Table 4).

Linearity—calibration range. Calibration curves of farnesol and tyrosol were measured in the concentration range 10–1000 nM, using seven calibration points (1000, 500, 200, 100, 50, 20 and 10 nM). For both analytes the response was linear in tested concentration range ($r^2 > 0.9994$) as it can be seen in Table 4. The repeatability of calibration curve was expressed as the repeatability of calibration curve slope in % of RSD of three measurements. The repeatability was about 5% for both analytes.

Limits of detection and quantitation (LOD and LOQ) were calculated based on S/N ratio. They were established first using standard solutions in mobile phase by the injection of the smallest amounts which provided S/N = 3 for LOD and S/N = 10 for LOQ. Subsequently this was confirmed by measurements in real matrix, which provided similar values. The results are displayed in Table 4. The method had good sensitivity to be able to perform the determination of farnesol and tyrosol in biological samples reaching LOQ

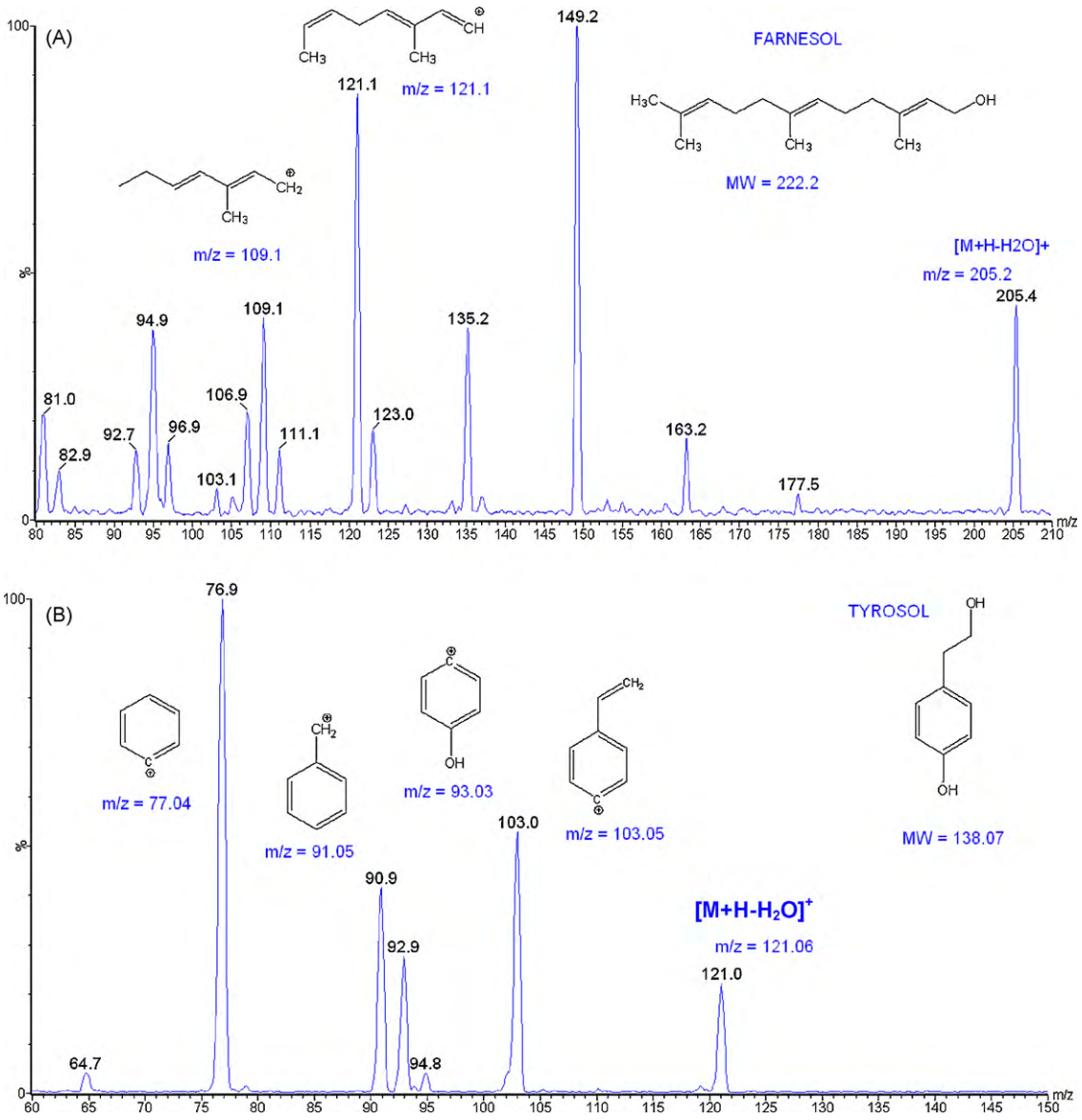

Fig. 3. Product ion spectra of farnesol (A) and tyrosol (B) measured on Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm) using acetonitrile:formic acid 0.075% (75:25) as a mobile phase at flow-rate 0.20 ml/min.

Table 4
The results of SST, validation results for linearity, sensitivity, accuracy and precision.

SST	Tyrosol	Farnesol
t_R [min]	1.24	3.85
Repeatability t_R [% RSD]	0.36	0.09
Repeatability A [% RSD]	3.30	2.40

Method validation		Tyrosol	Farnesol
Linearity [r^2]		0.9994	0.9998
Average slope of calibration curve		2.14	2.12
Repeatability of slope [% RSD]		5.47	4.46
LOQ [nM]		50	10.0
LOD [nM]		15.2	3.0
Accuracy [%]	L1	83.2	106.0
	L2	78.9	104.0
	L3	82.6	94.1
Precision [% RSD]	L1	1.36	3.77
	L2	1.43	3.71
	L3	3.28	0.50

L1, L2, and L3, concentration level 1 (1000 nM), level 2 (500 nM), level 3 (100 nM).

50 nM for tyrosol and 10 nM for farnesol and LOD 15.2 nM for tyrosol and 3.0 nM for farnesol.

Recovery and precision. Recovery and precision were established by spiking vaginal washing samples at three concentration levels of calibration curve—at high (1000 nM) medium (500 nM) and low (100 nM) concentration using sample preparation step described in Section 2.5. Method precision was determined as intra-day variability of three determinations at three different concentration levels expressed as % RSD, see Table 4. Intra-day precision was generally within 4% RSD for both analytes farnesol and tyrosol. Method accuracy ranged from 94.1 to 106.0% for farnesol and 78.9–83.2% for tyrosol.

Method selectivity–matrix effects. Matrix effects and method selectivity were tested using procedure described in Section 2.6. Neither negative nor positive peaks were observed at retention times of both analytes, therefore no matrix effects were observed and the method was found to be selective using UPLC–MS/MS in connection with protein precipitation sample pre-treatment step. High method selectivity was ensured using two SRM transitions and secondary ion ratio calculation.

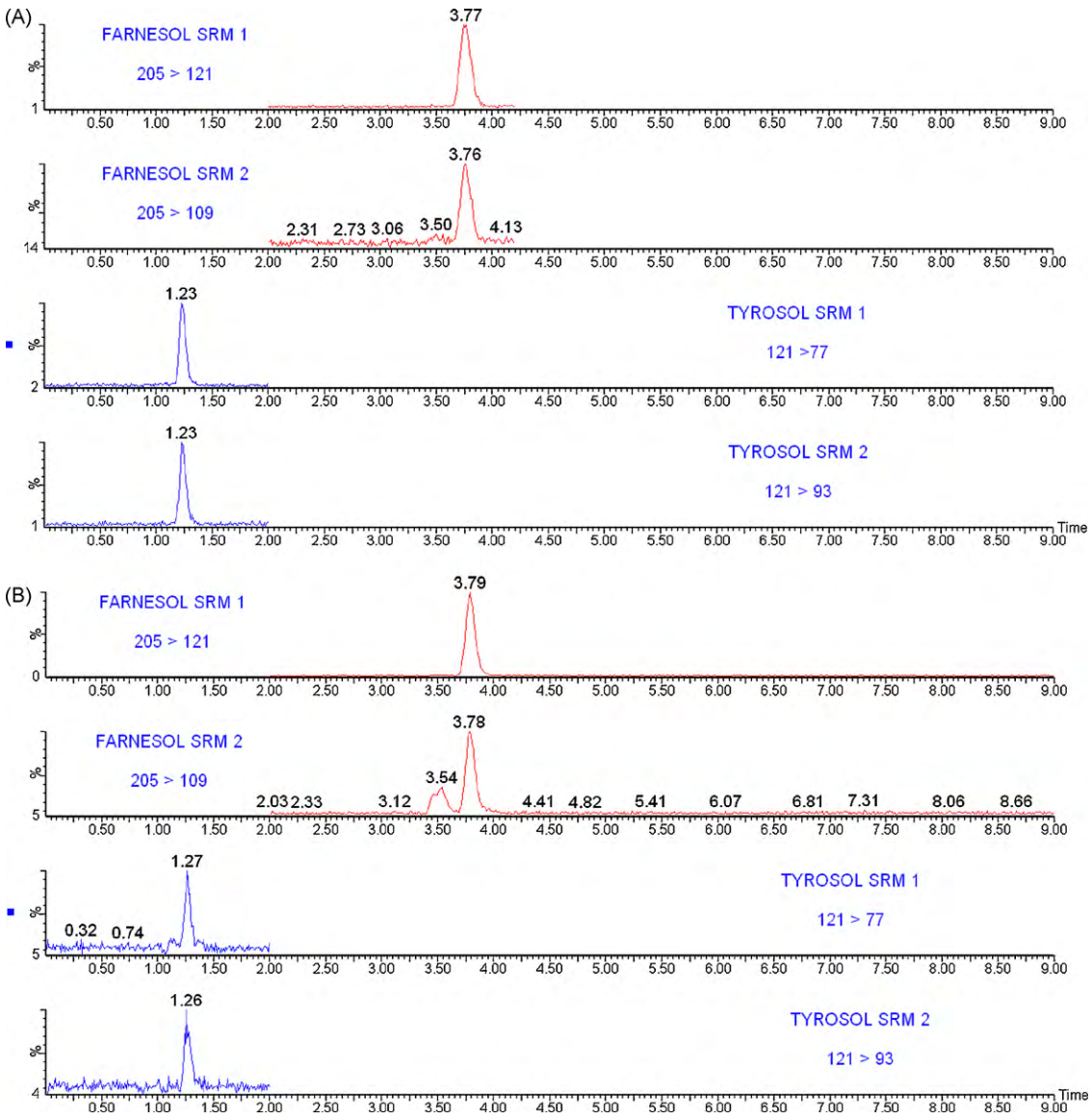

Fig. 4. UHPLC–MS/MS chromatogram of standard mixture of farnesol and tyrosol (calibration level 5×10^{-7} M) (A) and of vaginal washing sample (B) measured on Acquity BEH C18 (100 mm × 2.1 mm, 1.7 μm) using acetonitrile:formic acid 0.075% (75:25) as a mobile phase at flow-rate 0.20 ml/min.

3.4. Application to real samples

The samples of vaginal washing were measured using developed UHPLC–MS/MS method. A typical chromatogram could be seen in Fig. 4 A—standard solution and B—biological sample (vaginal washing after deproteinization). Extracted SRM chromatogram is displayed for each transition—SRM1 (121 > 77 and 205 > 121), a quantitative one, and SRM 2 (121 > 93 and 205 > 109), a confirmatory one. In the chromatogram of biological sample it is evident, that both QSM are present in tested sample. The peak in retention time 3.5 might possibly be a second isomer of farnesol, which does not possess QS activity, therefore it was not examined. Twelve samples from 10 women were evaluated for tyrosol and farnesol presence. Two women were negative for both of the compounds, one was positive only for farnesol, next one only for tyrosol, the rest ($n = 6$) was positive for both farnesol and tyrosol. The range QSM in positive individuals was 128–3133 nM for farnesol and 237–1758 nM for tyrosol, respectively. Typical results demonstrated low level of farnesol connected with higher level of tyrosol or on the other hand, low level of tyrosol at LLOQ and relatively high level of farnesol (as presented in Fig. 4B). Owing to low number of persons investigated, any general conclusions cannot be drawn for the moment.

Finding substantial variations in farnesol and tyrosol levels demonstrated the developed UHPLC–MS/MS method to be sufficiently sensitive and convenient for the purposes of evaluation of these QSM in vaginal washings. In order to confirm the structures in biological samples unequivocally, product ion scan and precursor ion scan measurements were used (data not shown) besides SRM experiments. Both product and precursor ion scans were in a good agreement with those of standard solutions. Also the calculations of secondary ion ratio were in a good agreement for standard solutions and real samples.

4. Conclusions

UHPLC–MS/MS method for the simultaneous determination of farnesol and tyrosol was developed. The method was fast (see Tables 1 and 2 for comparison of run times), sensitive and selective and it was proven to be applicable for the analysis of biological samples. MS/MS detection utilized two SRM transitions for each compound to ensure high selectivity and reliability of the method.

The method was validated according to the requirements of ICH with good results for linearity (>0.9994), precision (RSD < 4% for both analytes) and accuracy (79–106%). Analytes could be quantified at nM concentrations with typical LOQ 10 nM for farnesol and 50 nM for tyrosol. Compared to previously published methods, the sensitivity of our newly developed method for farnesol expressed as instrumental LOD was 9 fM, while the only reported sensitivity was 30 fM [13]. The sensitivity for tyrosol expressed as LOD was 2.07 ng/ml, while previously published results demonstrated sensitivity within the range LOD = 0.24–2000 ng/ml. The sensitivity was satisfactory for the intended purpose, as monitored QSM were present in biological samples at sufficient concentrations.

Applicability of the method was successfully tested in vaginal washings and provided results which can be helpful in the study of role of quorum-sensing molecules in potentially pathogenic yeasts.

Acknowledgements

The authors gratefully acknowledge the financial support of Research Center LC 531 and MSM 0021620822 of the Ministry of Education, Czech Republic.

References

[1] J.M. Hornby, E.C. Jensen, A.D. Lisec, J.J. Tasto, B. Jahnke, R. Shoemaker, P. Dussault, K.W. Nickerson, Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol, Appl. Environ. Microbiol. 67 (2001) 2982–2992.
[2] H. Chen, M. Fujita, Q. Feng, J. Clardy, G.R. Fink, Tyrosol is a quorum-sensing molecule in *Candida albicans*, PNAS 101 (2004) 5048–5052.
[3] K.B. Oh, H. Miyazawa, T. Naito, H. Matsuoka, Purification and characterization of an autoregulatory substance capable of regulating the morphological transition in *Candida albicans*, PNAS 98 (2001) 4664–4668.
[4] L. De Sordi, F.A. Mühlschlegel, Quorum sensing and fungal–bacterial interactions in *Candida albicans*, a communicative network regulating microbial coexistence and virulence, FEMS Yeast Res. 9 (2009) 990–999.
[5] M. Kruppa, Quorum sensing and *Candida albicans*, Mycoses 52 (2009) 1–10.
[6] L.S. Derengowski, C. De Souza-Silva, S.V. Braz, T.M. De Sousa, S.N. Báo, C.M. Kyaw, I. Silva-Pereira, Antimicrobial effect of farnesol, a *Candida albicans* quorum sensing molecule, on *Paracoccidioides brasiliensis* growth and morphogenesis, Ann. Clin. Microbiol. Antimicrob. 8 (2009), doi:10.1186/1476-0711-8-13, Article 13.
[7] N.C. Reading, V. Sperandio, Quorum sensing: the many languages of bacteria, FEMS Microbiol. Lett. 254 (2006) 1–11.
[8] D.A. Hogan, A. Vik, R. Kotler, A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology, Mol. Biol. 54 (2004) 1212.
[9] D.A. Hogan, Talking to themselves: autoregulation and quorum sensing in fungi, Eukaryot. Cell 5 (2006) 613–619.
[10] H. Lee, S. Finckbeiner, J.S. Yu, D.F. Škemer, T. Eisner, A.B. Attygalle, Characterization of (*E*,*E*)-farnesol and its fatty acid esters from anal scent glands of nutria (*Myocastor coypus*) by gas chromatography–mass spectrometry and gas chromatography–infrared spectrometry, J. Chromatogr. A 1165 (2007) 136–143.
[11] A.A. Khalil, B. Davies, N. Castagnoli, Isolation and characterization of a monoamine oxidase B selective inhibitor from tobacco smoke, Bioorg. Med. Chem. 14 (2006) 3392–3398.
[12] K. Teshima, T. Kondo, Analytical method for determination of allylic isoprenols in rat tissues by liquid chromatography/tandem mass spectrometry following chemical derivatization with 3-nitrophtalic anhydride, J. Pharm. Biomed. Anal. 47 (2008) 560–566.
[13] A.G. Staines, P. Sindelar, M.W.H. Coughtrie, B. Burchell, Farnesol is glucuronidated in human liver, kidney and intestine in vitro, and is a novel substrate for UGT2B7 and UGT1A1, Biochem. J. 384 (2004) 637–645.
[14] K. Torre-Carbot, O. Jauregui, E. Gimeno, A.I. Castellote, R.M. Lamuela-Raventos, M.C. Lopez-Sabater, Characterization and quantification of phenolic compounds in olive oils by solid-phase extraction, HPLC–DAD, and HPLC–MS/MS, J. Agric. Food Chem. 53 (2005) 4331–4340.
[15] M.J. Oliveras-Lopez, M. Innocenti, C. Giaccherini, F. Ieri, A. Romani, N. Mulinacci, Study of the phenolic composition of spanish and italian monocultivar extra virgin olive oils: distribution of lignans, secoiridoidic, simple phenols and flavonoids, Talanta 73 (2007) 726–732.
[16] K. Torre-Carbot, J.L. Chávez-Servín, O. Jaúregui, A.I. Castellote, R.M. Lamuela-Ravenós, M. Fitó, M.I. Covas, D. Munoz-Aguayo, M.C. López-Sabater, Presence of virgin olive oil phenolic metabolites in human low density lipoprotein fraction: determination by high-performance liquid chromatography–electrospray ionization tandem mass spectrometry, Anal. Chim. Acta 583 (2007) 402–410.
[17] K. Torre-Carbot, O. Jauregui, A.I. Castellote, M.R. Lamuela-Raventós, M.I. Covas, I. Casals, M.C. López-Sabater, Rapid high-performance liquid chromatography–electrospray ionization tandem mass spectrometry method for qualitative and quantitative analysis of virgin olive oil phenolic metabolites in human low-density lipoproteins, J. Chromatogr. A 1116 (2006) 69–75.
[18] F.N. Bazoti, E. Gikas, A.L. Skaltsounis, A. Tsarbopoulos, Development of a liquid chromatography–electrospray ionization tandem mass spectrometry (LC–ESI MS/MS) method for the quantification of bioactive substances present in olive oil mill wastewaters, Anal. Chim. Acta 573–574 (2006) 258–266.
[19] M.A.S. Alem, M.D.Y. Oteef, T.H. Flowers, L.J. Douglas, Production of tyrosol by *Candida albicans* biofilms and its role in quorum sensing and biofilm development, Eucaryot. Cell 10 (2006) 1770–1779.
[20] E. Fragopoulou, T. Nomikos, H.C. Karanonis, C. Apostolakis, E.P.M. Samiotaki, G. Panayotou, S. Antonopoulou, Biological activity of acetylated phenolic compounds, J. Agric. Food Chem. 55 (2007) 80–89.
[21] E. Miro-Casas, M.F. Albaladejo, M.I. Covas, J.O. Rodriguez, E.M. Colomer, R.M.L. Raventos, R. de la Torre, Capillary gas chromatography mass spectrometry quantitative determination of hydroxytyrosol and tyrosol in human urine after olive oil intake, Anal. Biochem. 294 (2001) 63–72.
[22] T. Watanabe, A. Yamamoto, S. Nagai, S. Terabe, Simultaneous analysis of tyrosol, tryptophol and ferulic acid in commercial sake samples by micellar electrokinetic chromatogramy, J Chromatogr. A 825 (1998) 102–106.
[23] European Pharmacopoeia 5th edition (Ph. Eur. 5), Council of Europe, Strasbourg, 2004.
[24] United States Pharmacopoeia 32, United States Pharmacopoeial Convention, Rockville, MD 20852, United States, 2009.
[25] International Conference on Harmonization (ICH), Q2 (R1): Text on Validation of Analytical Procedures, US FDA Federal Register (2003).