JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Evaluation of the Antimicrobial Activity of Citrus Essences on *Saccharomyces cerevisiae*

NICOLETTA BELLETTI, MAURICE NDAGIJIMANA, CLAUDIO SISTO, MARIA E. GUERZONI, ROSALBA LANCIOTTI, AND FAUSTO GARDINI*

Dipartimento di Protezione e Valorizzazione Agroalimentare, Università degli Studi di Bologna, Via Fanin 46, 40127 Bologna, Italy

The aim of this research was to assess the antimicrobial activity of nine different industrial essences used in a soft drink factory in relation to their composition, as well as to verify the role of vapor pressure on their bioactivity. The essences were tested against a *Saccharomyces cerevisiae* strain isolated from spoiled soft drinks. The tests were carried out by adding the essences directly to a liquid medium or into the headspace of closed systems inoculated with the yeast. The headspace composition was evaluated through a solid hase microextraction−gas chromatography technique. The use of a mass spectrometer allowed the identification of the peaks detected. The microbial growth was indirectly monitored by measuring the metabolic CO_2 released by the yeast. The results obtained indicated that the most effective essences were characterized by the highest concentration of some terpenes, such as citral, β-pinene, and *p*-cymene. Moreover, all of the essences were more bioactive when added directly to the liquid medium.

KEYWORDS: Citrus essences; terpenes; antimicrobial activity; *Saccharomyces cerevisiae*

INTRODUCTION

In recent years, the interest in the possible use of natural alternatives to food additives to prevent bacterial and fungal growth has notably increased. Plants and plant products can represent a source of natural alternatives to improve the shelf life and the safety of food. Also, they are characterized by a wide range of volatile compounds, some of which are important flavor quality factors (*1*). Recently, the interest in the application of essential oils to control plant and postharvest pathogens has increased and their potential role in food preservation has been exploited (*2*, *3*).

Plant essential oils have been studied for their antimicrobial activity against many microorganisms including several pathogens (*4*, *5*). In particular, the activity of oils from *Labiatae* (*6*−*9*) and citrus fruits (*10*, *11*) has been investigated. In addition, the action of single constituents of these oils has been exploited in order to better understand the cell targets of these molecules, to identify the most active molecules, and to balance the intrinsic variability of essential oils (*2*, *12*).

Commercial citrus essential oils are generally obtained from different parts of the plants by cold pressing (peels) or distillation (leaves) (*13*). Although fresh fruits and juices are the main commercial products of citrus fruits, their essential oils can also be used as ingredients in the pharmaceutical industry as well as in perfumery. In the food industry, essential oils and the essences derived from their concentrations are widely used as flavor enhancers in soft drinks, alcoholic beverages, and fruit-based products. In some cases, the composition of the flavoring essences can play an active role in the microbiological stability of the products. In fact, a recent industrial spoilage case, which involved more than 500000 orangeade bottles, has been attributed to the type of orange flavor added to the soft drinks (*14*). Citrus essential oils can have a very pronounced antimicrobial activity, even if their complexity and variability make it difficult to correlate their action to a specific component, as well as the possibility of antagonistic and synergistic effects. In general, the antimicrobial effects of essential oils have been mainly explained through the presence of C10 and C15 terpenes with aromatic rings and a phenolic hydroxylic group able to form hydrogen bonds with active sites of target enzymes (*5*, *7*, *15*, *16*). Nevertheless, other active terpenes, as well as alcohols, aldehydes, and esters, can contribute to the overall antimicrobial effects of the essential oils (*17*). For this reason, Caccioni et al. (*11*) proposed a holistic approach to explain the antimicrobial capabilities of citrus essential oils, whose performances could be the result of a certain quantitative balance of various components.

The aim of this research was to assess the inhibitory activity of nine different industrial essences used in a soft drink factory. These essences are used to flavor different kinds of soft drinks and can be added to the beverages at concentrations higher than 1000 ppm. Their antimicrobial activity was tested against the yeast strain *Saccharomyces cerevisiae* SPA, which was responsible for the spoilage episode described earlier (*14*). It is well-known that several procedures have been adopted to evaluate

* To whom correspondence should be addressed. Tel: +390512096575. Fax: +390512096573. E-mail: fgardini@kaiser.alma.unibo.it.

10.1021/jf049444v CCC: $27.50
Published on Web 10/16/2004

the antimicrobial activity of essential oils and that the data collected depend on the method used. In fact, each methodology can measure different aspects of microbial cell damage. In addition, the screening conditions can considerably affect the bioactivity of these substances (*18*−*20*). To evaluate yeast growth kinetics in relation to essence concentration, a gas chromatographic (GC) method was used. This method is based on the detection of metabolic CO_2 released from the microbial cells in the headspace of sealed vials. This indirect index of microbial growth has been extensively used to monitor microbial dynamics in food and beverages, as well as in model systems (*21*).

MATERIALS AND METHODS

Microbial Strain. The strain *S. cerevisiae* SPA was used in this study. It was isolated from industrial spoiled orangeades (*14*). The strain was maintained on Sabouraud dextrose agar (SDA) (Oxoid, Basingstoke, United Kingdom). Before the experiments, it was cultured in sabouraud dextrose broth (SDB, Oxoid) for 48 h at 28 °C.

Citrus Essence. Nine industrial citrus essences were considered in this research. Five of them were different orange-based essences (sweet orange, red orangeade, bitter orange, red orange, and Sicily orange) while the others were derived from Mandarin, lemon, sweet lime, and citron. Only two essences (sweet lime and red orangeade) were obtained by alcoholic extraction of the correspondent essential oils, while the other seven essences were derived from concentrations of the essential oils. All of the essences used were supplied by Flavorint (Messina, Italy).

Determination of the Antimicrobial Activity. The antimicrobial activity of the essences was tested on *S. cerevisiae* SPA inoculated in liquid or solid medium. To evaluate the antimicrobial effect in liquid medium, vials (10 mL capacity) containing 5 mL of SDB were inoculated with the target microorganism at an initial cell density of about 5×10^4 cfu/mL. The essences were added at different concentrations (300, 500, 1000, 1500, 3000, and 5000 ppm) solubilized in a constant amount of ethanol (5000 ppm). The vials were sealed with butyl septa and incubated at 28 °C. Inoculated vials containing only ethanol (5000 ppm) were used as the control.

The evaluation of the antimicrobial activity in solid media was carried out using 10 mL vials, containing 5 mL of SDA superficially inoculated (5×10^4 cfu/vial) with the target microorganism. The cells were inoculated at the interface between solid and vapor phase, and the aroma compounds were added directly in the headspace. The same concentrations of essences used in the liquid medium solubilized in the same amount of ethanol (5000 ppm) were introduced into the vials by soaking a 6 mm diameter filter paper disk (Antibiotica-Testblattchen, Shleicher and Schull, Dassel, Germany), which was fastened to the end of a tiny steel hook applied to the internal part of the butyl septa of the vials. This ensured that the paper disk did not touch the medium. In the control samples, the paper disk was impregnated with the same amount of ethanol used for the trial with liquid medium. The vials were closed and incubated at 28 °C.

CO_2 Determination. During the incubation, microbial growth was monitored by GC analysis of the metabolic CO_2 evolved in the headspace of the sealed vials. This automatic gas sampling is not destructive and allows several analyses of the same vials over time (*18*). A gas chromatograph GC 6000 Vega Serie 2 (Carlo Erba Instruments, Milan, Italy), equipped with a hot wire detector and 2 m × 2 mm i.d. glass-packed column filled with Porapak QS 80/100 mesh was used for CO_2 detection in the headspace of vials. The conditions for the analysis were as follows: column temperature, 100 °C; injector temperature, 100 °C; detector temperature, 180 °C; filament temperature, 230 °C; carrier gas (He) flow rate, 40 mL/min. For each analysis, 3.5 min was required. The gas chromatograph was connected to a Headspace Autosampler (model HS250, Carlo Erba Instruments) equipped with a gas syringe Gastight 1750 (Hamilton, Bonaduz, Switzerland). During sampling, the vials were maintained in the autosampler at 28 °C, and for each analysis, 0.2 mL of headspace was sampled. For each sample, three repetitions were considered. The variability coefficients never exceeded 5%. No variance analysis was performed because it is generally not required if biological variation within a treatment (coefficient of variation as standard deviation divided by mean) is lower than 10% and the difference among treatments is greater than three standard deviations. The R value of the Gompertz equation obtained was also higher than 0.920.

Data Analysis. The CO_2 percentage (v/v) in the headspace was analyzed over time and modeled according to the Gompertz equation as modified by Zwietering et al. (*22*):

$$y = A \exp\left\{ - \exp\left[\left(\frac{\mu_{max} e}{A}\right)(\lambda - t) + 1\right]\right\}$$

where y is the CO_2 percentage at time t, A is the maximum percentage of CO_2 produced, μ_{max} is the maximum CO_2 production rate (as $\Delta\%$ CO_2/h), and λ is the lag time (h) for CO_2 production. To evaluate the influence of the different concentrations of essence on the yeast growth, the efficacy index (EI) was calculated as follows:

$$EI = \frac{(\lambda_r - \lambda_c)}{\lambda_r} \times 100$$

where λ_r is the lag time (h) for CO_2 production in treated samples and λ_c is the lag time for CO_2 production (h) in the controls (without essences but containing ethanol).

GC-Mass Spectrometry (MS)-Solid Phase Microextraction (SPME) Analysis. A divinylbenzene−poly(dimethylsiloxane)-coated stable flex fiber (65 μm) and a manual SPME holder (Supelco Inc., Bellefonte, PA) were used in this study after preconditioning according to the manufacturer's instruction manual. Before each headspace sampling, the fiber was exposed to the GC inlet for 5 min for thermal desorption at 250 °C in a blank run. Five milliliters of the sample was placed in 10 mL vials, and the vials were sealed by PTFE/silicon septa. The samples were then equilibrated for 15 min at 70 °C. The SPME fiber was exposed to each sample for 5 min by manually penetrating the septum, and finally, the fiber was inserted into the injection port of the GC for 5 min sample desorption.

GC-MS analyses were carried out on an Agilent 6890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled to an Agilent 5970 mass selective detector operating in electron impact mode (ionization voltage, 70 eV). A Chrompack CP-Wax 52 CB capillary column (50 m length, 0.32 mm i.d., 1.2 μm df) was used (Chrompack, Middelburg, The Netherlands). The temperature program was 50 °C for 2 min, then programmed at 1 °C/min to 65 °C and finally at 5 °C/min to 220 °C, which was maintained for 22 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were performed with a split ratio of 1:20 and helium (1 mL/min) as the carrier gas. The compounds were identified by use of the National Institute of Standards and Technology−United States Environmental Protection Agency−National Institute of Health (*23*) and Wiley (*24*) mass spectra libraries as well as literature MS data and, whenever possible, coinjections with authentic chemical compounds. The results are reported as gas chromatographic peak area percentage.

RESULTS

Headspace Composition of the Extracts. Citrus essential oils and their derivatives are mainly terpene mixtures. It is well-known that many of these terpenes can have antimicrobial activities (*5*, *17*). In fact, such molecules can interact with some cellular structures causing the inhibition of cell growth or cell death. A preliminary condition for these effects is the contact between the antimicrobial molecule and the target cells. The contact usually requires the molecules to be in their most hydrophobic state, i.e., in their vapor phase, because this makes possible their solubilization in the cell membranes. For this reason, the composition of the headspace of the nine citrus essences studied in this research was analyzed. Although the headspace composition does not correspond to the whole essence

Table 1. Composition of the Head Space of the Nine Essences as Revealed by the HS-GC SPME Technique[a]

	essence								
constituent	sweet orange	red orangeade	lemon	citron	red orange	mandarin	sweet lime	bitter orange	Sicily orange
monoterpenes									
α-pinene	1.69	0.75	7.79	3.36	1.90	5.11	2.23	9.31	3.98
α-thujene	*b*					2.33			
sabinene				0.00	1.30			5.33	2.57
camphene				0.04		0.01	0.17		
β-pinene	0.22	0.17	21.28	20.07	1.48	3.40	8.78	1.13	0.46
β-phellandrene	1.06				1.15	0.54	1.27		
3-carene	0.39				0.42			1.46	
β-myrcene	4.38	0.95	4.04	2.24	4.01	3.61	1.16	2.71	12.30
α-terpinene					0.04	0.44			
4-carene			0.43	0.10					
limonene	76.87	49.25	42.30	41.07	72.68	34.62	44.27	61.06	56.85
β-thujene	9.80	0.24	2.10	0.78	12.22	13.47	0.60		16.19
cis-β-ocimene			0.21	0.07	0.08				
trans-β-ocimene								0.39	0.14
γ-terpinene	1.17	0.26	14.00	8.35	0.91	26.60	0.74	0.79	0.50
p-cymene	0.12	0.05	3.39	5.92	0.28	6.10	8.45	0.38	0.25
terpinolene	0.07		0.84	0.33	0.10	1.13	0.40	0.02	0.22
dehydro-*p*-cymene						0.03			
total	*95.78*	*51.68*	*96.37*	*82.32*	*96.58*	*97.38*	*68.07*	*82.57*	*93.46*
oxygenated monoterpenes									
1,4-cineol							0.55		
linalool oxide				0.04					
(*Z*)-limonene oxide			0.03	0.28		0.09		0.36	
(*E*)-sabinene hydrate				0.02	0.02	0.00		0.05	
(*Z*)-sabinene hydrate						0.05			
p-epoxy menth-8-ene									0.10
p-cymen-8-ol						0.06	0.07		
(*E*)-limonene oxide						0.08		0.26	0.14
epoxy terpinolene						0.06			
citronellal			0.10	0.27	0.08			0.43	
neral		0.11	0.53	2.49	0.12		0.39	0.03	0.08
geranial		0.20	0.82	4.65	0.23		0.58	0.49	0.16
perillal				0.10		0.07			
linalool	1.05	0.17	0.10	1.73	0.77	0.25		4.14	1.91
fenchol							0.12		
4-terpineol			0.05	0.31	0.07	0.13	0.15		
α-terpineol	0.15	0.07	0.10	0.83	0.14	0.38	1.30	0.58	0.28
borneol							0.08		
geraniol	0.12			0.27	0.03				
nerol					0.03				0.04
1-carveol						0.04		0.36	
cuminol				0.03					
thymol						0.14			
total	*1.32*	*0.55*	*1.73*	*11.00*	*1.47*	*1.36*	*3.24*	*6.70*	*2.71*
sesquiterpenes									
α-copaene		0.10							
farnesene			0.30			0.10			
bergamotene							0.21		
β-cubebene									0.14
germacrene D								0.14	0.04
caryophyllene			0.16	0.23	0.03		0.11	0.35	0.10
β-bisobolene				0.30			0.28		
valencene	0.19	0.05			0.15				1.39
cadinene					0.03			0.14	0.07
total	*0.19*	*0.15*	*0.46*	*0.53*	*0.21*	*0.10*	*0.60*	*0.63*	*1.74*
aliphatic alcohols									
ethanol		43.99	0.18	0.54	0.08		26.53		
hexanol									0.04
3-hexen-1-ol				0.14					
heptanol						0.04			
octanol	0.16				0.13			0.98	0.25
total	*0.16*	*43.99*	*0.18*	*0.69*	*0.21*	*0.04*	*26.53*	*0.98*	*0.29*
aliphatic aldehydes									
acetaldehyde		0.15							
octanal	0.54		0.09	0.31	0.50	0.22		4.93	0.40
nonanal	0.09		0.09	0.34	0.09	0.03		0.63	0.08
decanal	0.46			0.27	0.40	0.09		2.37	0.86
(*E*)-2-decenal									0.05
dodecanal								0.16	0.11
total	*1.09*	*0.15*	*0.18*	*0.92*	*0.99*	*0.34*		*8.09*	*1.50*

Table 1. (Continued)

constituent	essence								
	sweet orange	red orangeade	lemon	citron	red orange	mandarin	sweet lime	bitter orange	Sicily orange
esters									
ethyl butanoate		0.87	0.18						
ethyl hexanoate									
octyl acetate				0.16				0.17	
linalyl acetate		0.25		1.82				0.21	
neryl acetate			0.40	1.03	0.05				0.06
geranyl acetate			0.42	1.33					
neryl acetate									
methyl-methylanthranilate						0.76			
total		*1.11*	*1.00*	*4.34*	*0.05*	*0.76*		*0.37*	*0.06*
ketones									
6-methyl-5-hepten-2-one			0.04	0.16					
D-carvone						0.03		0.62	0.25
total			*0.04*	*0.16*		*0.03*		*0.62*	*0.25*
other compounds									
2-allyltoluene							0.10		
octanoic acid								0.03	
total							*0.10*	*0.03*	
total peak area[c]	2.3×10^8	1.6×10^8	8.8×10^9	2.8×10^8	1.3×10^8	1.0×10^8	9.8×10^7	1.3×10^8	1.4×10^8

[a] The data are reported as percentage of the area of each peak with respect to the total peak area. [b] Not detectable. [c] Arbitrary units.

components, it gives a measure of the volatile composition of the oil, which, in turn, depends on the vapor pressure of the molecules.

The headspace composition was evaluated through a SPME-GC technique. The use of a mass spectrometer allowed the identification of the peaks detected (*23*, *24*). **Table 1** reports the total area of the GC peaks and the percentage (on the basis of the relative peak area) of each compound present in the headspace, as well as the cumulative percentages of the classes of compounds (monoterpenes, sesquiterpenes, oxygenated monoterpenes, aliphatic alcohols, aliphatic aldehydes, esters, and ketones).

Limonene was the main constituent in all of the essences tested. In the orange-derived products (sweet orange, red orangeade, red orange, bitter orange, and Sicily orange), it accounted for percentages of the total peak area ranging between 56 and 77%. In general, β-thujene was quantitatively the second compound in the headspace of orange-based essences, with the exception of bitter orange in which relatively high amounts of α-pinene (9.3%) and linalool (4.14%) were found. Also, β-myrcene was present in noticeable amounts in sweet orange, red orangeade, red orange, and, especially, Sicily orange (12.3%).

In the other citrus essences, limonene accounted usually for about 40% of the total peak area of headspace. In the lemon essence, high quantities of β-pinene (21%), γ-terpinene (14%), and *p*-cymene (3.4%) were found. A similar situation was observed in sweet lime for β-pinene (8.8%) and *p*-cymene (8.4%), while γ-terpinene was not detected.

The headspace of Mandarin essence was characterized by a high concentration of β-thujene (13.5%), *p*-cymene (6.1%), and α-pinene (5.1%). However, the most abundant terpenes were limonene (34.6%) and γ-terpinene (26.6%).

Citron essence had the highest concentrations of compounds in the headspace, as demonstrated by the total peak area. Moreover, its composition was rather different from the other mixtures tested. β-Pinene accounted for 20% of the total area, γ-terpinene for 8.4%, and *p*-cymene for 5.9%. α-Pinene, β-phellandrene, and β-myrcene reached concentrations of about 3%. Moreover, this essence was characterized by a high concentration of two oxygenated monoterpenes: neral (2.5%) and geranial (4.6%), the isomers of citral. This essence was also characterized by the presence of a high amount of esters such as linalyl acetate, neryl acetate, and geranyl acetate. In the red orangeade and sweet lime samples, relevant amounts of ethanol were detected, due to the production procedure of these flavoring agents, which are alcoholic extracts.

Antimicrobial Activity of the Essences. The antimicrobial properties of the citrus essences were tested on the strain *S. cerevisiae* SPA. The growth of the yeast, both in liquid and in solid media, was monitored through the evaluation of the metabolic CO_2 evolved in the headspace of the sealed vials. The accumulation of this metabolite can be considered an indirect index of the microbial activity (*21*). The amount of CO_2 in the headspace accounts for the contribution of all of the cells in the system from the inoculum to the analysis time. The CO_2 data over time were fitted with the Gompertz equation as modified by Zwietering et al. (*22*), and the growth parameters A, μ_{max}, and λ were estimated. Previous work has demonstrated that the lag times estimated modeling CO_2 data through the Gompertz equation are very close to those estimated modeling cell load data expressed as CFU/g or mL (*21*). For this reason, enumeration of viable count was not included and the duration of λ was considered as the base to calculate the EI.

Figure 1 shows the curves obtained for different concentrations of citron essence added to the solid medium. The antimicrobial effects were expressed as a percentage increase of the λ phase length with respect to the sample in which only ethanol was present (EI). The EI values relative to the essences obtained both in liquid and in solid media are shown in **Table 2**.

If added to the liquid medium, all of the flavoring agents were able to increase the λ time by about 20%, when present at their lower concentrations, with the exception of the red orangeade essence. The increase in λ time of *S. cerevisiae* SPA caused by red orangeade did not reach 25% also when the higher concentrations were used. This value increased (50%) in sweet orange essence and was about 85% for red orange and 100% for bitter orange. Also, in the presence of 5000 ppm of Sicily orange, the λ time was more than doubled. Increasing amounts

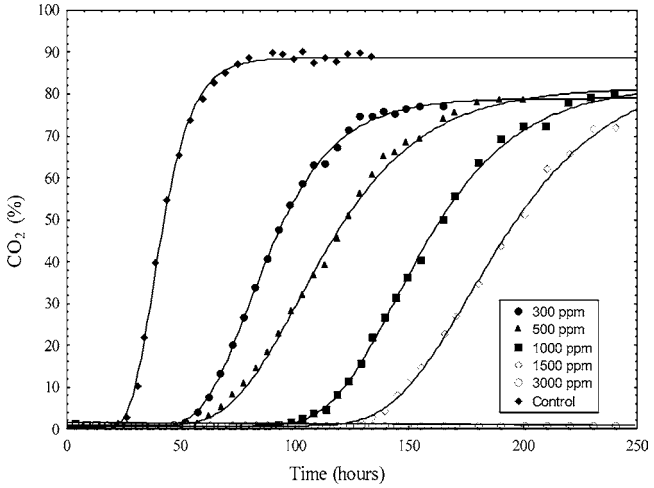

Figure 1. CO_2 evolution over time in sealed vials containing solid medium inoculated with *S. cerevisiae* SPA in the presence of different amounts of citron essence. The experimental data were modeled with the Gompertz equation as modified by Zwietering et al. (*22*).

of lemon essence progressively prolonged the λ time of *S. cerevisiae* SPA, and no growth was observed at a concentration of 5000 ppm after 240 h of incubation. The sweet lime essence increased the λ time by about 170% at a concentration of 3000 ppm and significantly prolonged the λ time when added at 5000 ppm (280%). Citron essence was the most effective. In fact, it completely inhibited the growth of *S. cerevisiae* SPA when present at 500 ppm, while the presence of 300 ppm caused a remarkable increase in the λ time (71%).

Similar behaviors were observed when the citrus essences were added to the headspace of solid media inoculated with the target organism. Also, in these experimental conditions, citron was the most effective essence followed by sweet lemon and lemon. Also, Mandarin essence doubled the lag time of *S. cerevisiae* when added at a concentration of 1000 ppm. However, the λ time increases were, at the same concentrations, noticeably lower than those observed in liquid media. In fact, citron essence completely inhibited yeast growth only when added in the HS at a level of 3000 ppm, while sweet lemon at 5000 ppm tripled the λ time but did not inhibit the growth as observed in the liquid medium. A reduced antimicrobial efficacy was observed also for the other citrus extracts.

DISCUSSION

The overall antimicrobial activity of complex mixtures, such as the citrus essences tested in this research, cannot easily be explained through the action of a single or few molecules, and a holistic approach can be more suitable to evaluate the inhibition of microbial growth (*11*). Nevertheless, the most active essences were characterized by a high concentration of specific molecules, such as β-pinene, *p*-cymene, and citral isomers.

β-Pinene was found in high amounts (more than 20%) in the headspace of lemon and citron essences, but also, in sweet lime, it accounted for more than 9% of the total peak area. In contrast, in Mandarin essence, it only reached a concentration of 3.4%, while in the orange-based essences it was usually present at levels lower than 1%. The negative effect of β-pinene on the yeast membrane function has been already studied by Uribe et al. (*25*). It is also known to have antioxidant and estrogenic activities (*23*) as well as spasmolytic activity (*27*). This terpene was found to be particularly effective against *Escherichia coli* O157:H7 (*28*). Moreover, β-pinene is one of the most important components of a sage essential oil, which was able to reduce the growth kinetics of many microorganisms (*29*). In addition, the bridged bicyclic terpenes, α- and β-pinene, had a relevant inhibitory activity against many yeasts and molds, with β-pinene being the most active (*30*). These findings were supported by some authors (*31*), while other studies indicated a reduced activity for these molecules (*32*−*34*).

Citral (the mixture of the isomers neral and geranial) was found in a remarkable concentration (more than 7%) in citron

Table 2. Effect on the Growth of *S. cerevisiae* of the Various Citrus Essences Added to Solid and Liquid Media at Different Concentrations[a]

		liquid medium		solid medium	
essence	concn (ppm)	λ (h)	efficacy index[b]	λ (h)	efficacy index
control[c]		28.80 (0.18)[d]	0.00	28.40 (0.09)	0.00
red orangeade	300	28.99 (0.23)	0.66	28.21 (0.11)	−0.67
	500	32.02 (0.41)	11.18	29.40 (0.49)	3.52
	1000	31.21 (0.08)	8.37	28.47 (0.13)	0.25
	1500	31.00 (0.07)	7.64	31.95 (0.66)	12.50
	3000	34.38 (0.51)	19.38	32.54 (0.69)	14.58
	5000	35.71 (0.65)	23.99	34.75 (0.54)	22.36
bitter orange	300	34.50 (0.12)	19.79	30.06 (0.44)	5.85
	500	33.32 (0.70)	15.69	29.24 (0.12)	2.96
	1000	37.87 (0.44)	31.49	32.23 (0.22)	13.49
	1500	54.13 (1.16)	87.95	37.26 (0.36)	31.20
	3000	59.27 (0.82)	105.70	39.66 (0.45)	39.65
	5000	62.13 (1.21)	115.73	40.81 (0.28)	43.74
citron	300	49.36 (0.30)	71.39	59.55 (0.40)	109.68
	500	ng[e]		72.02 (0.97)	153.59
	1000	ng		115.24 (1.45)	305.77
	1500	ng		142.62 (1.62)	402.18
	3000	ng		ng	
	5000	ng		ng	
mandarin	300	42.56 (0.55)	47.78	29.11 (0.15)	2.50
	500	50.87 (1.01)	76.63	32.44 (0.26)	14.23
	1000	58.27 (0.79)	102.33	34.78 (0.54)	22.46
	1500	57.81 (1.07)	100.73	43.21 (0.32)	52.15
	3000	59.01 (0.90)	104.90	50.21 (0.41)	76.80
	5000	68.17 (1.25)	136.70	64.81 (0.88)	128.20
Sicily orange	300	36.84 (0.15)	27.92	27.90 (0.17)	−1.76
	500	37.67 (0.68)	30.80	32.18 (0.59)	13.31
	1000	42.48 (0.86)	47.50	31.18 (0.26)	9.79
	1500	43.67 (0.41)	51.63	30.89 (0.48)	8.77
	3000	48.70 (0.53)	69.10	42.59 (0.22)	49.96
	5000	58.22 (0.16)	102.15	42.26 (0.30)	48.80
lemon	300	35.07 (0.81)[d]	21.77	29.42 (0.09)	3.59
	500	35.48 (0.72)	23.19	32.85 (0.82)	15.67
	1000	42.98 (0.58)	49.24	37.66 (0.26)	32.61
	1500	49.90 (0.94)	73.26	40.45 (0.68)	42.43
	3000	70.28 (1.19)	144.03	54.01 (1.50)	90.18
	5000	ng[e]		71.33 (0.84)	151.16
sweet lime	300	33.59 (0.22)	16.63	27.57 (0.10)	−2.92
	500	33.66 (0.41)	16.88	30.24 (0.10)	6.48
	1000	35.61 (0.12)	23.65	35.43 (0.29)	24.75
	1500	53.09 (0.89)	84.34	32.29 (0.69)	13.70
	3000	78.12 (1.23)	171.25	48.17 (1.02)	69.61
	5000	110.24 (1.85)	282.78	92.17 (1.69)	224.54
red orange	300	38.47 (0.46)	33.58	30.43 (0.42)	7.15
	500	42.22 (0.07)	46.60	30.46 (0.29)	7.25
	1000	44.20 (0.61)	53.47	32.79 (0.58)	15.46
	1500	45.24 (0.81)	57.08	31.59 (0.76)	11.23
	3000	48.50 (0.15)	68.40	36.36 (0.22)	28.03
	5000	53.52 (0.94)	85.83	42.62 (1.02)	50.07
sweet orange	300	39.20 (0.84)	36.11	24.41 (0.25)	−14.05
	500	39.50 (0.23)	37.15	33.13 (0.64)	16.65
	1000	40.10 (0.65)	39.24	45.52 (0.61)	60.28
	1500	41.23 (0.55)	43.16	45.92 (0.91)	61.69
	3000	42.08 (0.48)	46.11	46.18 (0.24)	62.61
	5000	45.16 (0.92)	56.81	47.02 (0.65)	65.56

[a] The λ time for CO_2 production of the control in the absence of essence is also reported. [b] Percentage increase of the lag time of the sample with essence with respect to the control without essence. [c] Sample inoculated in the absence of essences. [d] Standard deviation. [e] No growth observed within 240 h.

essence, whose antimicrobial activity was the highest among the tested mixtures. Moreover, in this essence, a high percentage of acetic esters of neral and geranial was found. Citral was found effective against citrus postharvest pathogens by Wuryatmo et al. (*35*). Ben Yehoshua et al. (*36*, *37*) and Rodov et al. (*38*) postulated a significant relationship between the presence of citral in the peel of citrus fruits and the reduction of their decay caused by *Penicillium digitatum*. Also, Caccioni and Deans (*39*) and Caccioni et al. (*40*) found this mixture of isomers to have the highest antifungal activity as compared with other components of citrus essential oils. The inhibitory effects of citral against microorganisms are due to the presence of a carbonyl group adjacent to the α- and *β*-carbons in the α,*β*-unsaturated aldehydes neral and geranial (*32*). This makes the *β*-carbon positively polarized and the aldehyde can act as a direct alkylating agent able to bind cellular nucleophilic groups (*41*).

In Mandarin essence, a high concentration (more than 8%) of *p*-cymene was found. A high amount of this terpene characterized also the headspace of lemon, citron, and sweet lime (3.4, 5.9, and 8.5%, respectively), while it was found in negligible amounts in the orange-based products. However, *p*-cymene alone was among the less bioactive compounds of thyme essential oil (*32*). Similar results were observed by Bagamboula et al. (*42*) on *Shigella sonnei* and *Shigella flexneri.* Also, Dorman and Deans (*5*), in their wide screening on the antimicrobial activity of volatile molecules, found *p*-cymene to be among the less effective. Otherwise, the use of *p*-cymene in combination with carvacrol enhanced the inhibion of *Bacillus cereus* (*43*).

All of the orange-based products (characterized by high concentrations of limonene, *β*-thujene, *β*-myrcene, and linalool) tested in this research showed a lower inhibiting effect against the target yeast. In a wide screening on the antifungal activity of citrus essential oils, Caccioni et al. (*11*) also observed a weaker antifungal activity of orange essential oil against *P. digitatum* and *Penicillium italicum* with respect to other citrus essential oils. The reduced antimicrobial action can be explained by the different headspace composition. In fact, Caccioni et al. (*40*) found a significant correlation between the antimicrobial effects of citrus essential oil and the amount of some minor monoterpenes hydrocarbon (monoterpenes other than limonene), total monoterpenes, and sesquiterpenes.

Even the most effective essences tested in this research showed a lower antimicrobial activity when added to the headspace. To explain the higher efficacy of the citrus extracts in a liquid medium, several hypotheses can be proposed as follows: (i) The solubilization of the active molecules in the aqueous phase of the solid medium does not allow their contact with the microbial cells located on the surface of the medium; (ii) the molecules released faster into the headspace could not be the most effective; and (iii) the surface exposure to the active molecules of cells superficially inoculated on the solid medium is lower as compared to that of a cell suspended in a liquid medium; this latter effect may be enhanced by the inoculum procedure, which favors the clumping of cells, further reducing the amount of exposed surface.

Although only two essences were able to completely inhibit the *S. cerevisiae* SPA growth, some of the tested essences showed interesting potentials as antimicrobials in food systems and beverages, taking also into account the high inoculum level considered in this research, which does not match the contamination of real foods, such as soft drinks and beverages. The optimization of the use of these substances needs a deeper study on the effects that the main chemical and physical factors of a food system (i.e., temperature, pH, a_w, composition, structure, etc.) can play on their bioactivity. A wide amount of literature indicates that the antimicrobial activity of molecules such the terpenes present in citrus extracts is dependent on their ability to reach the cell targets (in first instance the cell membranes) and, consequently, on their vapor pressure. In the vapor phase, in fact, these molecules are characterized by higher hydrophobicity and can be solubilized in the membrane lipidic bilayer. Thus, the possible ways to reinforce the antimicrobial activity of aroma compounds could be (i) a calibrated increase of their vapor pressure in order to enhance their capacity to interact with the microbial cell membranes, through the use of specific solutes or active packaging able to slowly release the bioactive molecules; (ii) their use in combination with other hurdles to microbial growth; and (iii) the identification of the most active molecules and set up of calibrated mixtures in which the bioactive constituents are present at constant levels.

Independent of the strategy adopted, focusing on the most active molecules and their mechanisms of action becomes fundamental for a practical application of these natural substances. To reach this goal, standardization of the methods used to test the antimicrobial efficacy of essential oils or their derivatives seems to be a prerequisite. Also, Suhr and Nielsen (*44*) observed that appropriate screening procedures are needed to relate to the potential future applications. The lack of agreement observed between screening procedures and real foods studies can be explained by the fat and protein contents of the food as well as by the mobility of the active molecules within the system and by their probability of meeting the target cells.

In conclusion, in the evaluation of the microbial inhibition due to a complex mixture of compounds, such as essential oils, the most important antimicrobial molecules should be identified and their eventual interactions studied. Such an approach could be useful to increase the control of microbial growth, to minimize the impact of these substances on the flavor of food products, and to avoid fluctuations in essential oils activity due to meteorological, seasonal, and geographical factors, as well as different compositions due to the plant type.

LITERATURE CITED

(1) Utama, I. M. S.; Willis, R. B. H.; Ben-Yehoshua, S.; Kuek, C. In vitro efficacy of plant volatiles for inhibiting the growth of fruit and vegetable decay microorganisms. *J. Agric. Food Chem.* **2002**, *50*, 6371−6377.

(2) Vázquez, B. I.; Fente, C.; Franco, C. M.; Vázquez, M. J.; Cepeda, A. Inhibitory effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. *Int. J. Food Microbiol.* **2001**, *67*, 157−163.

(3) Lanciotti, R.; Gianotti, A.; Patrignani, F.; Belletti, N.; Guerzoni, M. E.; Gardini, F. Use of natural aroma compounds to improve shelf life and safety of minimally processed fruits. *Trends Food Sci. Technol.* **2004**, *15*, 201−208.

(4) Delaquis, P. J.; Stanich, K.; Girard, B.; Mazza, G. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *Int. J. Food Microbiol.* **2002**, *74*, 101−109.

(5) Dorman, H. J. D.; Deans, S. G. Antimicrobial agents from plant: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* **2000**, *88*, 308−316.

(6) Basilico, M. Z.; Basilico, J. C. Inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production. *Lett. Appl. Microbiol.* **1999**, *29*, 238−241.

(7) Juven, B. J.; Kanner, J.; Sched, F.; Weisslowicz, H. Factors that interact with the antibacterial of thyme essential oil and its active constituents. *J. Appl. Bacteriol.* **1994**, *76*, 626−631.

(8) Lambert, R. J. W.; Skandamis, N.; Coote, P. J.; Nychas, G.-J. E. A study of minimum inhibitory concentration and mode of actions of oregano essential oil, thymol and cavacrol. *J. Appl. Microbiol.* **2001**, *91*, 453−462.
(9) Tassou, C.; Koutsounanis, K.; Nychas, G.-J. E. Inhibition of *Salmonella enteritidis* and *Staphylococcus aureus* in nutrient broth by mint essential oil. *Food Res. Int.* **2000**, *33*, 273−280.
(10) Ben-Yehoshua, S.; Kim, J. J.; Shapiro, B. Curing of citrus fruit. Applications and mode of action. In *5th International Controlled Atmosphere Research Conference, June 14−16*; Fellman, G. K., Ed.; Wenatchee, Washington, 1989; Vol. 2, pp 179−195.
(11) Caccioni, D. R. L.; Guizzardi, M.; Biondi, D. M.; Renda, A.; Ruberto, G. Relationships between volatile components of citrus fruit essential oils and antimicrobial action on *Penicillium digitatum* and *Penicillium italicum*. *Int. J. Food Microbiol*. **1998**, *43*, 73−79.
(12) Karatzas, A. K.; Bennik, M. H. J.; Smid, E. J.; Kets, E. P. W. Combined action of S-carvone and mild heat treatment on *Listeria monocytogenes* Scott, A. *J. Appl. Bacteriol.* **2000**, *89*, 296−301.
(13) Lota, M.-L.; de Rocca Serra, D.; Tomi, F.; Jacquemond, C.; Casanova, J. Volatile components of peel and leaf oils of lemon and lime species. *J. Agric. Food. Chem.* **2002**, *50*, 796−805.
(14) Ndagijimana, M.; Belletti, N.; Lanciotti, R.; Guerzoni, M. E.; Gardini, F. Effect of aroma compounds on the microbial stabilization of orange-based soft drinks. *J. Food Sci.* **2004**, *69*, 20−24.
(15) Farag, R. S.; Daw, Z. Y.; Hewedy, F. M.; El-Baroti, G. S. A. Antimicrobial activity of some Egyptian spice essential oils. *J. Food Prot.* **1989**, *52*, 665−667.
(16) Daferera, D. J.; Ziogas, B. N.; Polissiou, M. G. GC-MS analysis of essential oils from some Greek aromatic plants and their fungitoxicity on *Penicillium digitatum*. *J. Agric. Food. Chem.* **2000**, *48*, 2576−2581.
(17) Sikkema, J.; de Bont, J. A. M.; Poolman, B. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **1995**, *59*, 201−222.
(18) Skandamis, P. N.; Tsigarida, E.; Nychas, G.-J. E. Ecophysiological attributes of *Salmonella typhimurium* in liquid culture and within a gelatin gel with or without the addition of oregano essential oil. *World J. Microbiol. Biotechnol.* **2000**, *16*, 31−35.
(19) Skandamis, P. N.; Nychas, G.-J. E. Effect of oregano essential oil on microbiological and physicochemical attributes of minced meat stored in air and modified atmospheres. *J. Appl. Microbiol.* **2001**, *91*, 1011−1022.
(20) Skandamis, P. N.; Nychas, G.-J. E. Preservation of fresh meat with active and modified atmosphere packaging conditions. **2002**, *79*, 35−45.
(21) Gardini, F.; Lanciotti, R.; Sinigaglia, M.; Guerzoni, M. E. A headspace gas chromatographic approach for the monitoring of the microbial cell activity and the cell viability evaluation. *J. Microbiol. Methods* **1997**, *29*, 103−114.
(22) Zwietering, M. H.; Jongenberger, J.; Rombouts, F. M.; van't Riet, K. Modeling of the bacterial growth curve. *Appl. Environ. Microbiol.* **1990**, *56*, 1785−1881.
(23) National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health [NIST/EPA/NIH]. *Mass Spectral Database, Standard Reference Database No 1A*, version 1.6; NIST/EPA/NIH: Gaithersburg, MD, 1998.
(24) *Registry of Mass Spectral Data*; Wiley: New York, 1998.
(25) Uribe, S.; Ramirez, J.; Peña, A. Effects of β-pinene on yeast membrane functions. *J. Bacteriol.* **1985**, *161*, 1195−1200.
(26) Perry, N. S.; Houghton, P. J.; Sampson, J.; Theobald, A. E.; Hart, S.; Lis-Balchin, M.; Hoult, J. R.; Evans, P.; Jenner, P.; Milligan, S.; Perry, E. K. In vitro activity of *Salvia Lavandulaefolia* (Spanish sage) relevant to treatment of Alzheimer's disease. *J. Pharm. Pharmacol.* **2001**, *53*, 1347−1356.
(27) Sadraei, H.; Asghari, G. R.; Hajhashemi, V.; Kolagar, A.; Ebrahimi, M. Spasmolytic activity of essential oil and various extracts of *Ferula gummosa* Boiss on ileum contractions. *Phytomedicine* **2001**, *3*, 370−376.
(28) Takikawa, A.; Abe, K.; Yamamoto, M.; Ishimaru, S.; Yasui, M.; Okubo, Y.; Yokoigawa, K. Antimicrobial activity of nutmeg against *Escherichia coli* O157. *J. Biosci. Bioeng.* **2002**, *94*, 315−320.
(29) Tepe, B.; Daferera, D.; Sokmen, A.; Sokmen, M.; Polissiou, M. Antimicrobial and antioxidant activities of the essential oil and various extracts of *Salvia Tomentosa* Miller (Lamiaceae). *Food Chem.* In press.
(30) Hammer, K. A.; Carson, C. F.; Riley, T. V. Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. *J. Appl. Microbiol.* **2003**, *95*, 853−860.
(31) Adegoke, G. O.; Iwahashi, H.; Komatsu, Y.; Obuchi, K.; Iwahashi, Y. Inhibition of food spoilage yeasts and aflatoxinogenic moulds by monoterpenes of the spice *Aframomum danielli*. *Flavour Fragrance J.* **2000**, *15*, 147−150.
(32) Cosentino, S.; Tuberoso, C. I. G.; Pisano, B.; Satta, M.; Mascia, V.; Arzedi, E.; Palmas, E. In-vitro antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Lett. Appl. Microbiol.* **1999**, *29*, 130−135.
(33) Raman, A.; Weir, U.; Bloomfield, S. F. Antimicrobial effects of tea-tree oil and its major components on *Staphylococcus aureus*, *Staph. epidermidis* and *Propionibacterium acnes*. *Lett. Appl. Microbiol.* **1995**, *21*, 242−245.
(34) Mourey, A.; Canillac, N. Anti-*Listeria monocytogenes* activity of essential oil components of conifers. *Food Control* **2002**, *13*, 331−336.
(35) Wuryatmo, E.; Klieber, A.; Scott, E. S. Inhibition of citrus postharvest pathogens by vapor of citral and related compounds in culture. *J. Agric. Food. Chem.* **2003**, *51*, 2637−2640.
(36) Ben-Yehoshua, S.; Rodov, V.; Kim, J. J.; Carmeli, S. Preformed and induced antifungal materials of citrus fruit in relation to the enhancement of decay resistance by heat and untraviolet treatments. *J. Agric. Food Chem.* **1992**, *40*, 1217−1221.
(37) Ben-Yehoshua, S.; Rodov, V.; Fang, D. Q.; Kim, J. J. Preformed antifungal compounds of citrus fruits: effect of postharvest treatments with heat and growth regulators. *J. Agric. Food Chem.* **1995**, *43*, 1062−1066.
(38) Rodov, V.; Ben-Yehoshua, S.; Fang, D. Q.; Kim, J. J.; Ashkenazi, R. Preformed antifungal compounds of lemon fruit: Citral and its relation to disease resistance. *J. Agric. Food Chem.* **1995**, *43*, 1057−1061.
(39) Caccioni, D. R. L.; Deans, S. G. Action of citrus fruits essential oils on germination of *Penicillium digitatum* and *Penicillium italicum*. In *International Symposium on Industrial Crops and Products*, 22−24 November; Pisa, Italy, 1993.
(40) Caccioni, D. R. L.; Deans, S. G.; Ruberto, G. Inhibitory effect of citrus fruits essential oil components on *Penicillium italicum* and *Penicillium digitatum*. *Petria* **1995**, *5*, 177−182.
(41) Witz, G. Biological interactions of α,β-unsaturated aldehydes. *Free Radical Biol. Med.* **1989**, *7*, 333−349.
(42) Bagamboula, C. F.; Uyttendaele, M.; Debevere, J. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and *p*-cymene towards *Shigella sonnei* and *S. flexneri*. *Food Microbiol.* **2004**, *21*, 33−42.
(43) Ultee, A.; Slump, R. A.; Steging, G.; Smid, E. J. Antimicrobial activity of carvacrol toward *Bacillus cereus* on rice. *J. Food Prot.* **2000**, *63*, 620−624.
(44) Suhr, K. I.; Nielsen, P. V. Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *J. Appl. Microbiol.* **2003**, *94*, 665−674.

Received for review April 5, 2004. Revised manuscript received August 24, 2004. Accepted September 1, 2004.

JF049444V