



Modeling of combined effects of citral, linalool and β -pinene used against *Saccharomyces cerevisiae* in citrus-based beverages subjected to a mild heat treatment

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ABSTRACT

The aim of this work was to evaluate the antimicrobial activity of three terpenes (citral, linalool and β -pinene), in combination with a mild heat treatment (55 °C, 15 min). The study has been carried out on an orange based soft drink inoculated using a wild strain of *Saccharomyces cerevisiae*. The results, expressed as growth/no-growth data, were analyzed with the logistic regression. A model comprising only of significant individual parameters ($p \leq 0.05$) and describing the relationships between terpene concentrations and the probability of having stable beverages was obtained. When citral and β -pinene were combined, the citral concentration required to achieve a 50% probability of having stable bottles ($P = 0.5$) dropped from 100.9 $\mu\text{L/L}$ in the absence of β -pinene to 49.3 $\mu\text{L/L}$ in the presence of 20 $\mu\text{L/L}$ of β -pinene. The mixture of citral and linalool was less effective, in fact, the same probability ($P = 0.5$) was obtained combining 60 $\mu\text{L/L}$ of linalool with 35.1 $\mu\text{L/L}$ of citral. The addition of 20 $\mu\text{L/L}$ of linalool and β -pinene reinforced citral bioactivity and the concentration of citral needed to reach $P = 0.5$ fell from 100.9 $\mu\text{L/L}$ in the presence of citral alone to 42.0 $\mu\text{L/L}$. The presence of both linalool and β -pinene at a concentration of 40 or 60 $\mu\text{L/L}$ in the absence of citral led to a lower spoilage probability ($P = 0.58$ and $P = 0.93$, respectively). It can be concluded that the antimicrobial potential of the three terpenes alone can be strengthened combining appropriate concentrations of each of them. This study confirmed also the potentiating effect of a mild temperature treatment on the antimicrobial efficacy of the molecules. Neither the thermal treatment alone nor the presence of the terpenes at their maximum concentrations (without thermal treatment) were able to guarantee the microbial stability of the beverages.

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1. Introduction

The chemico-physical and product characteristics (low pH, high C/N ratio, and nutrients) of soft drinks make them susceptible to the growth of specific microbial groups, such as acetic and lactic acid bacteria, moulds, and yeasts (Battay et al., 2001, 2002). The addition of CO_2 in beverages further reduces the growth possibility mainly to yeasts (Louriero and Querol, 1999). The stability of these beverages often depends on thermal treatments to which ingredients, intermediate and final products can be subjected. However, some products cannot be thermally treated (because of the thermal damage of some packaging material or the thermal degradation of flavour compounds) and their stability relies upon the addition of weak acids or other preservatives. The effectiveness of these antimicrobials depends on several factors, among which the most important are pH, microbial cell concentration, and the intrinsic resistance to the antimicrobials of the microorganisms present after bottling (Steels et al., 2000; Warth, 1988).

The producers are, however, searching for alternatives to such preservatives because they are perceived by consumers as extraneous and not natural. This consumer perception has been strengthened also by the cases recently reported that benzene originated from benzoic acid in soft drinks through chemical reactions (Gardner and Lawrence, 1993; Federal Institute for Risk Assessment, 2005; United Kingdom Food Standards Agency, 2006; Meadows, 2006).

In this scenario, the search for new strategies and new antimicrobials for beverage (and other products) stabilization becomes a central goal for producers. Aroma compounds and essential oils can be an interesting alternative. Their antimicrobial potential is well known (Burt, 2004; Dorman and Deans, 2000; Fisher and Phillips, 2008; Holley and Patel, 2005; Kalemba and Kunicka, 2003). A key role of orange essential oil in the microbial stability of orangeade has been reported by Ndagijimana et al. (2004), while Fitzgerald et al. (2004) described the function of vanillin in inhibiting microbial degradation of beverages. In addition, Belletti et al. (2007) demonstrated the possibility to prevent microbial growth in uncarbonated beverages combining a mild thermal treatment with an essential oil or aroma compounds used at concentrations compatible with the sensorial profile of the product. Otherwise, the main limitations to an industrial use of these substances as preservatives are

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their organoleptic impact and the variable composition of the essential oils (which can be reflected in their antimicrobial activity) (Burt, 2004; Lanciotti et al., 2004). The action of single constituents of the essential oils has been studied to identify the most active molecules to balance the intrinsic variability of essential oils (Karatzas et al., 2000; Vázquez et al., 2001). Also, the combination of essential oils with other stabilizing treatments has been proposed to reduce their organoleptic impact and to standardize the product safety and shelf-life requirements. In addition, the combination of essential oils or their components with a mild heat treatment can reduce the impact of the latter on the final quality and costs of the products (Alzamora and Guerriero, 2003). Since the use of combined hurdles to inhibit microbial growth can result in their additive, antagonistic and synergic interactions, predictive microbiology is an invaluable tool to determine accurately the shelf life and stability of food products. In this framework, logistic regression models are gaining importance (Zhao et al., 2001), for the probabilistic modeling of dichotomous data (for example growth/no-growth, toxin produced/toxin not produced). Based on empirical data, logistic regression calculates the probability of a binary outcome as a linear function of a combination of predictor variables (Hosmer and Lemeshow, 1989). Recently this approach was useful to assess the probability of stability of beverages (Battey et al., 2002, 2001; Belletti et al., 2007). The results obtained with a citron essential oil were particularly interesting because it is commonly used as flavouring agent in soft drinks (Belletti et al., 2007). The antimicrobial activity of this oil was confirmed *in vitro* (Belletti et al., 2004) and in fruit salads (Belletti et al., 2008), and was related to the high concentration of citral, whose antimicrobial potential is known, as reported by other authors (Rivera-Carriles et al., 2005; Wuryatmo et al., 2003). However, the overall bioactivity of an essential oil is the result of the bioactivity of the single constituents, whose effects can be additive, synergistic or antagonistic (Alzamora and Guerriero, 2003; Kalemba and Kunicka, 2003; Rivera-Carriles et al., 2005; Santiesteban-López et al., 2007). The complexity of the composition of citrus essential oils induced Caccioni et al. (1998) to propose a holistic approach to explain the antimicrobial capabilities of essential oils, whose performances could be the result of a certain quantitative balance of various components.

In this work the microbial stability of an uncarbonated orange based beverage has been evaluated in relation to the presence of 3 terpenes, i.e. citral, linalool and β -pinene. Citral is a mixture of two isomers, geranial and neral, which are acyclic α,β -unsaturated monoterpene aldehydes naturally occurring in many essential oils from citrus fruits or other herbs or spices (Friedman et al., 2004; Tzortzakakis and Economakis, 2007; Wuryatmo et al., 2003). The antimicrobial action exerted by citral against yeasts and moulds in different conditions has already been demonstrated (Belletti et al., 2007, 2008; Caccioni and Deans, 1993; Rivera-Carriles et al., 2005; Wuryatmo et al., 2003).

Linalool is an oxygenated monoterpene present in the oil of several plants and fruits, such as citrus, basil, coriander, lavender, etc. (Fisher and Phillips, 2008; Krist et al., 2008; Suppakul et al., 2003; Wan et al., 1998). A good bioactivity against several microorganisms of this terpene was reported by Kotan et al. (2007) and Krist et al. (2008), while Bagamboula et al. (2004) found a low antimicrobial activity of linalool against *Shigella sonnei* and *Sh. flexneri*.

β -pinene is a bicyclic terpene and is a common constituent, together with its isomer α -pinene, of oils from Lamiaceae, conifers, citrus and many other plants (Belletti et al., 2004; Burt, 2004; Canillac and Mourey, 2001; Hong et al., 2004; Marino et al., 2001). It has been identified as one of the most important bioactive constituents of many essential oils (Aligiannis et al., 2001; Couladis et al., 2003). On the other hand, some authors found little or no antimicrobial activity (Filipowicz et al., 2003; Pichette et al., 2006).

The aim of this study was to evaluate if the antimicrobial activity of citral against a *Saccharomyces cerevisiae* strain previously observed in beverages (Belletti et al., 2007) was affected by the presence of the other two terpenes. The results expressed as growth/no-growth data observed in beverages after 60 days storage were analyzed with logistic

regression and through the logit transformation the probability of growth of the yeast and, consequently, its ability to spoil the beverages was obtained.

2. Materials and methods

2.1. Strain

S. cerevisiae SPA, the yeast strain used in this work, belongs to the strain collection of the Department of Food Science of the University of Bologna (Ndagijimana et al., 2004). The culture was maintained on slants of Sabouraud Dextrose Agar (SDA) (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) until usage. Before the experiments, it was cultured twice in Sabouraud Dextrose Broth for 48 h at 28 °C. After a presumptive quantification of the culture with a Bürker chamber, serial dilutions were performed in order to obtain the yeast concentration desired (approx. 4 log cfu/bottle). A precise counting of the initial yeast concentration was carried out by plate count on SDA incubated at 28 °C for 72 h.

2.2. Experimental design

The experimental design used was a CCD with 3 variables (citral, linalool and β -pinene concentration) at 5 levels reinforced by the addition of 5 combinations, located in the boundaries of growth/no-growth of yeast (runs 18–22). The variables and levels used are reported in Table 1.

2.3. Preparation of beverages

The beverages were prepared by aseptically diluting an industrial citrus-based concentrate used for soft drink manufacturing (Flavourint, Madone, Italy) (dilution factor, 1:6; final, 8.5 °Bx). Five hundred ml PET bottles (Flavourint) were filled with the beverages. PET bottles were previously sanitized with a diluted hydrogen peroxide (3%) solution (Carlo Erba Reagents, Milan, Italy). Ten repetitions of each run of the experimental plan were prepared. Ten samples containing the maximum terpene concentration provided by the experimental plan but not thermally treated were also prepared. Before closing, the filled bottles were preheated at 55 °C in a water bath, and then supplemented with the aroma compounds dissolved in ethanol (Merck,

Table 1

Experimental design adopted for the evaluation of stability of the beverages and frequency of not spoiled bottles observed for each run.

Run	Aroma compound ($\mu\text{L/L}$)			Fraction of stable bottles
	Citral	β -pinene	Linalool	
1	30	15	15	0.3
2	90	15	15	1.0
3	30	45	15	0.8
4	90	45	15	0.9
5	30	15	45	0.7
6	90	15	45	1.0
7	30	45	45	1.0
8	90	45	45	1.0
9	60	30	30	0.9
10	60	30	30	0.9
11	0	30	30	0.2
12	120	30	30	1.0
13	60	0	30	0.1
14	60	60	30	1.0
15	60	30	0	0.9
16	60	30	60	1.0
17	60	30	30	0.9
18	0	0	0	0.0
19	120	60	60	1.0
20	0	15	15	0.0
21	30	0	15	0.0
22	30	15	0	0.0

Rome, Italy). Independently of the amount of flavouring agent added, the final concentration of ethanol was 0.5% (vol/vol). The same ethanol amount was added to the samples not supplemented with flavouring agents. Subsequently, the bottles were inoculated with $10^{4.4}$ cfu/bottle of *S. cerevisiae* SPA, corresponding to about 50 cfu/ml. Finally, they were immediately closed with screw caps and treated at 55 °C in the water bath for 15 min. After the heat treatment, samples were rapidly cooled at room temperature in a water/ice bath. The bottles were stored at room temperature (28 ± 3 °C) and observed periodically over a 60-day period for the presence of cloudiness, cell sediment on the bottom, and/or an evident swelling of the bottles due to yeast growth. After 60 days, all the no-growth results were confirmed by plating 0.1 ml of each bottle on SDA and incubating the plates at 28 °C for 5 days.

2.4. Models development

The value 1 was assigned to the bottles in which yeast growth was not observed while 0 meant that growth occurred. A logistic regression analysis was conducted on the raw data using Statistica 6.1 (StatSoft Italy srl, Vigonza, Italy) in order to assess the probability (*P*) of growth during the storage period as a function of the combinations of aroma compound concentration. The final model was obtained through a stepwise procedure and included only parameters with $p \leq 0.05$.

3. Results

In Table 1 the results, expressed as frequency of not spoiled bottles for each run of the experimental plan, are shown. The samples characterized by the absence of visible yeast growth after 60 days were plated onto SDA to check the presence of viable yeast cells which however were never found (cell load < 10 cfu/mL). The data of Table 1 show that in the absence of terpenes (run 18) the yeast grew in all the samples and indicate that the yeast could survive the mild thermal treatment applied, confirming a previous study (Belletti et al., 2007). Moreover, also the presence of the terpenes alone was not sufficient to avoid yeast growth. In fact, the bottle inoculated with the three terpenes added at the maximum concentration provided by the experimental design but not pasteurized were all spoiled (data not included in the experimental plan). In contrast, spoilage did not occur in any of the pasteurized bottles, with the three terpenes added at their highest levels (run 19).

The logit model was used to find relationships among the considered variables and the probability of having stable (not spoiled) beverages.

A second order polynomial equation was used to fit the observed data and a simplified model was obtained through a stepwise procedure which allowed considering only significant individual terms (with $p \leq 0.05$). In fact, as observed by Battey et al. (2002), model simplification is useful for several reasons. Complete models can have very high correlation coefficients and describe the data used to create the model with great accuracy, but this accuracy can penalize the prediction accuracy. This occurs because factors in these over-parameterized models are actually fitting noise in the data rather than the data themselves (Gauch, 1993).

In the simplified model, only four terms were considered, and namely two linear terms (citral and β -pinene) and two quadratic terms (β -pinene and linalool); the equation obtained was:

$$-6.6216 + 0.0656 \times [\text{citral}] + 0.2133 \times [\beta - \text{pinene}] - 0.0022 \times [\beta - \text{pinene}]^2 + 0.0012 \times [\text{linalool}]^2.$$

The *p* values were 2.396×10^{-8} , 2.313×10^{-7} , 5.063×10^{-4} , 4.398×10^{-2} , 1.665×10^{-3} for constant, citral, β -pinene, β -pinene² and linalool² respectively. The goodness of fit of the model was indicated by its relative chi-square (178.54 with $p < 0.00001$) and by the relationship between fitted and observed values for each run of the experimental design (Fig. 1), while the loss function (the squared value of the differences between observed and fitted values) of the simplified model was 51.89.

Fig. 2 shows the probability of stability of the bottles provided by the model when only one of the terpenes was added. In the absence of linalool and β -pinene, citral concentration increase resulted in a progressive inhibition of the yeast growth. The predicted spoilage probability was lower than 25% when citral was added at its higher concentration (120 $\mu\text{L/L}$). Also for β -pinene the predicted spoilage probability decreased with the increase of its concentration. However, the highest predicted yeast inhibition was observed with a β -pinene concentration of about 50 $\mu\text{L/L}$. Linalool, also when used at the highest concentration tested (60 $\mu\text{L/L}$), was quite ineffective assuring less than the 5% of sample stability. However, it should be considered that with a concentration of 60 $\mu\text{L/L}$ the stabilizing effect of citral considered alone is similar to that exerted by the same concentration of linalool.

Fig. 3 shows the cross effect of citral and β -pinene in the absence of linalool. The presence of just 20 $\mu\text{L/L}$ of β -pinene considerably affected yeast growth: in fact, a $P = 0.5$ was achieved with 100.9 $\mu\text{L/L}$ of citral

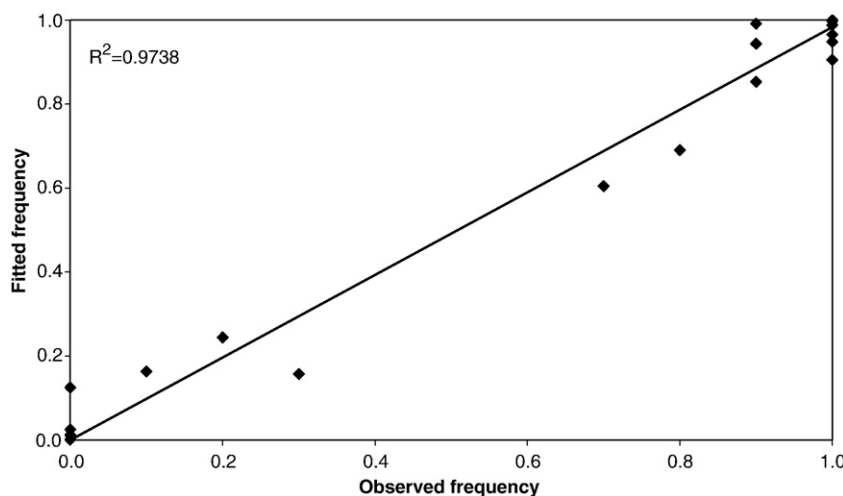


Fig. 1. Observed and fitted values of probability of not spoiled bottles for each run of the experimental plan obtained with the simplified model.

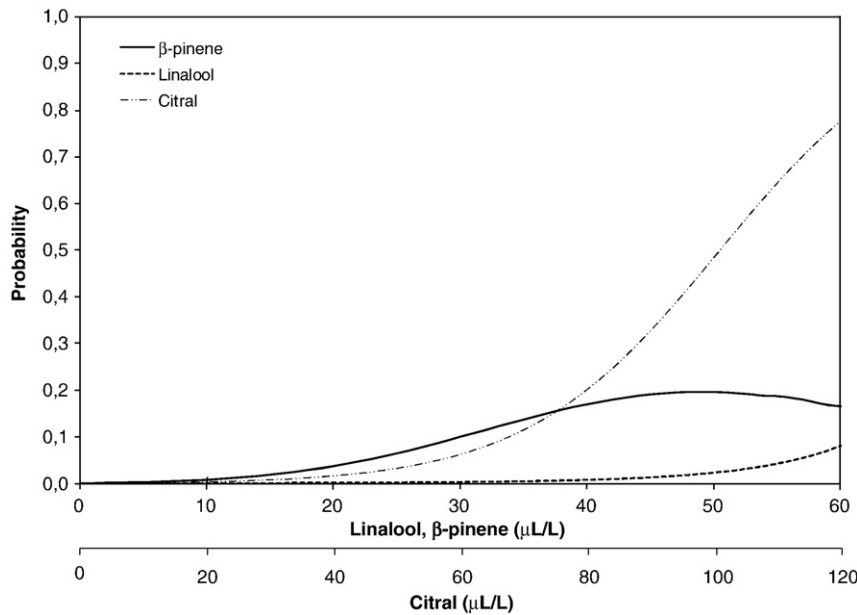


Fig. 2. Predicted probability of stability (P) provided by the simplified model for beverages inoculated with *S. cerevisiae* SPA ($10^{4.4}$ cfu/bottle) and subjected to a mild heat treatment (55 °C, 15 min). The effect of each molecule was considered alone, keeping constant at 0 $\mu\text{L/L}$ the concentration of the others in the model.

in the absence of β -pinene, or with 49.3 $\mu\text{L/L}$ of citral in the presence of 20 $\mu\text{L/L}$ of β -pinene. This concentration was further reduced (24.5 $\mu\text{L/L}$) in the presence of 40 $\mu\text{L/L}$ of β -pinene; however, successive increases of this terpene did not cause any further significant improvement of the microbial stability of beverages.

In contrast, as reported in Fig. 4, in the absence of β -pinene, 20 $\mu\text{L/L}$ of linalool caused a limited inhibition of yeast growth, while increasing concentration of this oxygenated monoterpene resulted in a more marked limitation of the possibility of yeast spoilage, and, in the presence of 60 $\mu\text{L/L}$ of linalool, only 35.1 $\mu\text{L/L}$ of citral are requested to have $P=0.5$.

Fig. 5 reports the concomitant effects of linalool and β -pinene on the antimicrobial activity of citral. The addition of 20 $\mu\text{L/L}$ of each of these terpenes reinforced citral bioactivity and the concentration of citral needed to reach $P=0.5$ fell from 100.9 $\mu\text{L/L}$ in the presence of citral alone to 42.0 $\mu\text{L/L}$. The increase of linalool and β -pinene concentrations caused a further reduction of the concentration of citral necessary to

have the same effect against *S. cerevisiae* SPA. Also, the presence of both linalool and β -pinene at a concentration of 40 or 60 $\mu\text{L/L}$ was sufficient to guarantee a lower spoilage probability ($P=0.58$ and $P=0.93$, respectively) even in the absence of citral.

The simplified model was used also to generate Fig. 6 which represents the concentrations of the three terpenes which correspond to a 90% stability ($P=0.9$). This figure shows that the inhibiting effect of citral is strongly reinforced by the addition of β -pinene up to 30 $\mu\text{L/L}$. After this threshold the effect of the increase of β -pinene concentration is negligible as indicated also by the inclusion in the final model of the quadratic term of β -pinene with a positive sign.

4. Discussion

Citral, β -pinene and linalool are common constituents of several citrus essential oils (Belletti et al., 2004; Fisher and Phillips, 2008) and

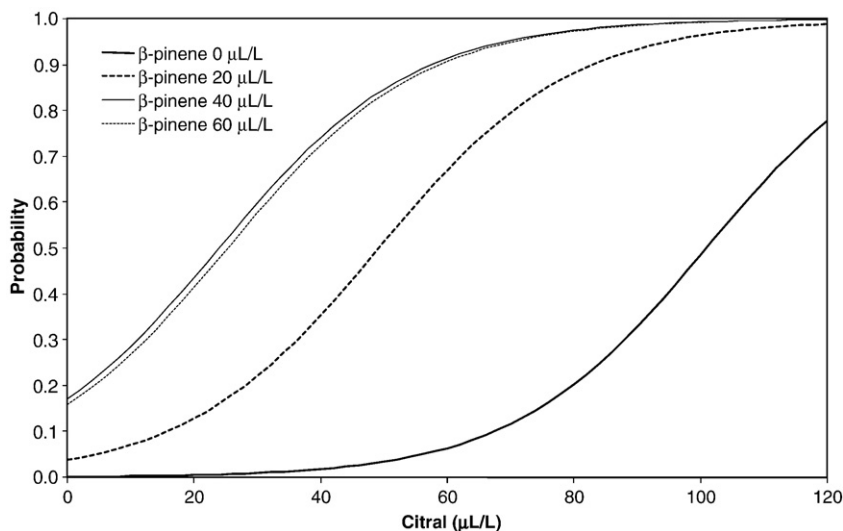


Fig. 3. Predicted probability of stability (P) provided by the simplified model for beverages inoculated with *S. cerevisiae* SPA ($10^{4.4}$ cfu/bottle) and subjected to a mild heat treatment (55 °C, 15 min). The concentration of linalool in the model was kept constant at 0 $\mu\text{L/L}$ and each curve was drawn for a defined concentration of β -pinene: 0 $\mu\text{L/L}$ (solid line), 20 (dotted line), 40 (dashed line) or 60 (thin dashed line) $\mu\text{L/L}$.

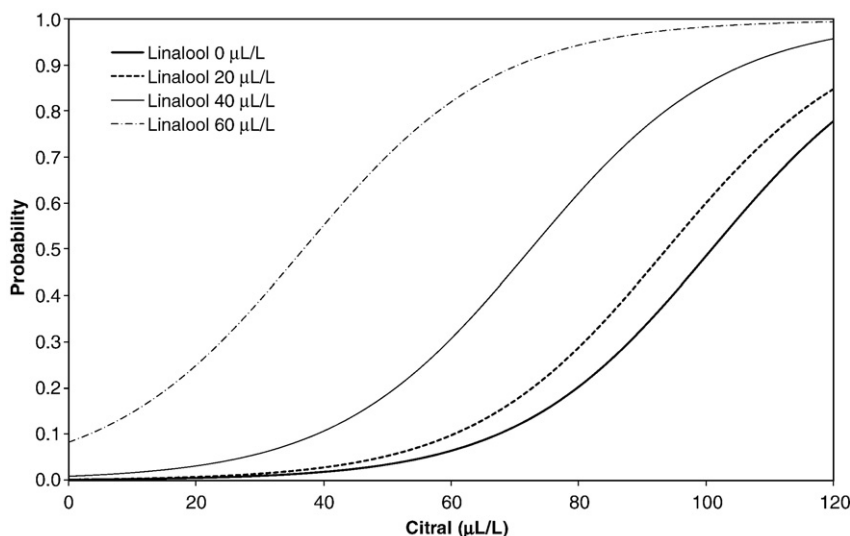


Fig. 4. Predicted probability of stability (P) provided by the simplified model for beverages inoculated with *S. cerevisiae* SPA ($10^{4.4}$ cfu/bottle) and subjected to a mild heat treatment (55°C , 15 min). The concentration of β -pinene in the model was kept constant at $0\ \mu\text{L/L}$ and each curve was drawn for a defined concentration of linalool: $0\ \mu\text{L/L}$ (solid line), 20 (dotted line), 40 (thin solid line) or 60 (thin dashed line) $\mu\text{L/L}$.

can have antimicrobial action. However this study showed that their antimicrobial activity can be notably potentiated throughout the combination of appropriate concentrations of each of them, focusing the attention on the cross antimicrobial effects of different constituents of essential oils, in agreement with the holistic approach proposed by Caccioni et al. (1998). In addition, this study confirmed also the relationship between the temperature treatment and the antimicrobial efficacy of the molecules. In fact, neither the thermal treatment alone nor the presence of the terpenes at their maximum concentrations in the absence of the thermal treatment was able to guarantee the microbial stability of the beverages. In this perspective, the inhibition of *S. cerevisiae* SPA growth observed can be considered the result of the cumulative damages caused by the sublethal thermal treatment and the presence of terpenes. The temperature treatment enhanced the bioactivity of terpenes increasing their vapour pressure and, in turn, their possibility to solubilize in the yeast cell membrane.

According to the model obtained, within the concentration ranges used here, citral considered alone was not able to achieve a microbial

stability of beverages higher than 90%. In addition, linalool and β -pinene at their maximum concentration ($60\ \mu\text{L/L}$) inhibited the yeast growth of less than 10% and 20% of the bottles, respectively. However, increasing amounts of β -pinene (up to about 40 – $50\ \mu\text{L/L}$ after which this molecule seems to have no further effect) or linalool or both of them, dramatically increased the microbial stability of the beverages in the presence of citral. Noteworthy, even in the absence of citral, the addition of the other two terpenes at a concentration of $60\ \mu\text{L/L}$ was able to reduce the possibility of growth to 10% of the samples ($P=0.9$).

According to Rivera-Carriles et al. (2005), these effects can be defined as synergistic, and are probably the result of the combination of the effects on membrane integrity and cytoplasm systems caused by molecules which can be characterized by different mode of action. In this case, the antimicrobial activity of citral has been explained by the fact that it is a member of the α,β unsaturated aldehydes which act as alkylating agent towards nucleophilic groups of essential cellular constituents (Witz, 1989; Wuryatmo et al., 2003); the antimicrobial effect of β -pinene has been studied by Uribe et al. (1985) and has been

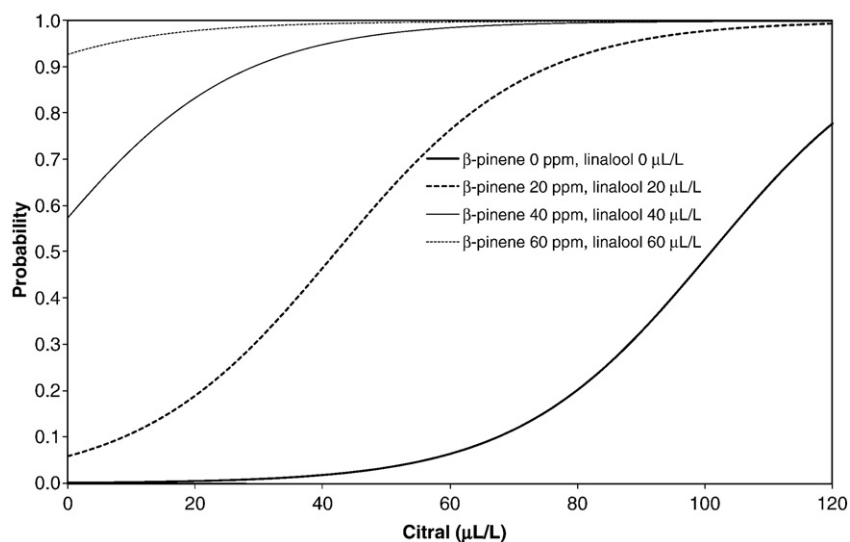


Fig. 5. Predicted probability of stability (P) provided by the simplified model for beverages inoculated with *S. cerevisiae* SPA ($10^{4.4}$ cfu/bottle) and subjected to a mild heat treatment (55°C , 15 min). Each curve was drawn for a defined concentration of linalool and β -pinene: $0\ \mu\text{L/L}$ (solid line), 20 (dashed line), 40 (thin solid line) or 60 (thin dotted line) $\mu\text{L/L}$ of each terpene.

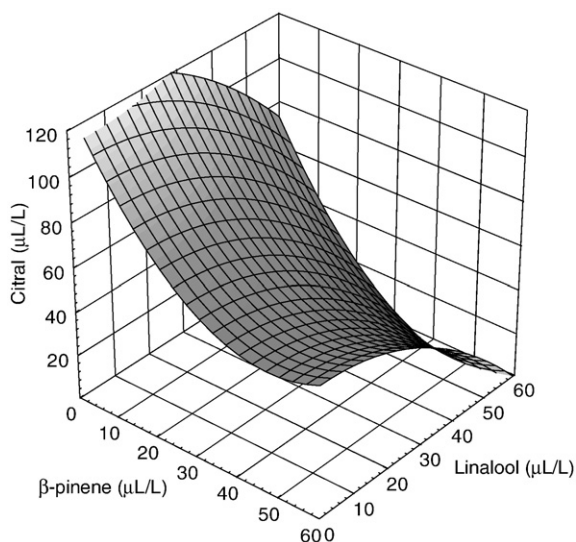


Fig. 6. Combination of the three terpenes able to assure a predicted probability of having 90% of stable bottles ($P=0.9$) according to the simplified model for beverages inoculated with *S. cerevisiae* SPA ($10^{4.4}$ cfu/bottle) and subjected to a mild heat treatment (55 °C, 15 min).

attributed to alterations produced at the level of the membranes. To our knowledge, there are no reports of the specific mechanism of action of linalool.

Synergism refers to an enhancement of antimicrobial activity of a compound because of the presence of a second compound (Rivera-Carriles et al., 2005). However, the presence of a true synergistic effect has been deeply debated. Recently Bidlas and Lambert (2008) stated that the use of the term “synergic” is often confusing and sometimes the synergistic relationships between variables can be due to a improper use of the models or to an incorrect interpretation of the physical interactions between factors. Concerning essential oils and their constituents synergistic effects have been described with chemico-physical factors, such as a_w , pH, salt as well as chelators and organic acids (Lachowicz et al., 1998; Lambert et al., 2004; López-Malo et al., 1998; Santiesteban-López et al., 2007; Zhou et al., 2007b). Moreover, while some authors found no synergy between some selected essential oil constituents (Gutierrez et al., 2009; Lambert and Lambert, 2003; Nazer et al., 2005), Rivera-Carriles et al. (2005) demonstrated a synergistic antimicrobial effect between citral and other phenolic compounds (vanillin, thymol, carvacrol, eugenol) and similar effects were described also by Kubo and Fujita (2001), Valero and Francés (2006) and Zhou et al. (2007a). Thus, if synergistic functions of the various molecules contained in an essential oil, in comparison to the action of one or two main components of the oil, seems questionable, it is possible that the activity of the major components is modulated by other minor molecules (Bakkali et al., 2008).

In this case, the antimicrobial activity of the three terpenes was further potentiated by the sublethal thermal treatment carried out. A similar effect has been already observed for S-carvone (Karatzas et al., 2000), cinnamon and clove essential oils (Knight and McKellar, 2007), carvacrol and cymene (Periago et al., 2004), as well as for (E)-2-hexenal, citral and citron essential oil (Belletti et al., 2007). The increase of temperature up to 55 °C increased the vapour pressure of the molecules enhancing their ability to solubilize in the plasma membrane of yeasts and, in turn, enhancing their bioactivity (Lanciotti et al., 2004).

In conclusion, the thermal treatment and the presence of the three terpenes considered alone were unable to avoid the spoilage of all the samples. The addition to beverages of increasing amounts of the three terpenes in combination with the thermal treatment progressively increased the frequency of not spoiled bottles. Finally, citral, linalool and

β -pinene strengthened each other their antimicrobial action when used in combination.

In this framework, the combination of a mild thermal treatment with the presence of some selected aroma compounds can be an important strategy to inhibit or to delay microbial growth in many food products avoiding the problems arising from the organoleptic impact of many terpenes. If high concentrations are required to achieve useful essential oil antimicrobial activity, unacceptable levels of inappropriate flavours and odors may result (Gutierrez et al., 2009). In fact, sometimes, the microbiological control through the use of essential oils and their constituents in real food is obtained by adding amounts of these substances (up to thousand $\mu\text{L/L}$) incompatible with an acceptable aroma profile. The concentrations of citral, β -pinene and linalool used in this work are well-matched with those added to a beverage through the addition of citrus essential oils usually used as ingredients at industrial level. At the same time, the reduction of the thermal treatment (in beverages such as those considered here it is usually carried out at 65–70 °C for several minutes) results in reduced energetic costs and higher nutritional and flavour characteristics of the final product.

A more effective application of this strategy for microbial stabilization of foods needs a deeper comprehension of how these agents act and affect microbial metabolisms. However, the knowledge of cross effects of the bioactive molecules on microbial stability of food is fundamental to optimize the use of these substances as antimicrobials in real food. The variability in essential oil composition is widely reported in literature (Burt, 2004) and a minimum concentration of the principal bioactive component should be requested for the use of essential oils in antimicrobial perspective. In other words, as suggested by Santiesteban-López et al. (2007), it is necessary to define “antimicrobial equivalent mixtures” able to guarantee a specific and constant inhibition of the microbial growth. In addition, also the eventual antagonistic effect between essential oil constituents has to be studied to avoid a dangerous decrease of their antimicrobial potential.

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