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The antifungal effect of 20 essential oils against the most important moulds in terms of spoilage of bakery products (*Eurotium* spp., *Aspergillus* spp. and *Penicillium* spp.) was investigated. Suitable solutions of essential oils were added directly to an agar culture medium (containing 2% wheat flour) to obtain a final concentration in the range between 0 to 1,000 ppm. Antifungal activity was tested at different water activity (a_w) and pH conditions, and the fungal growth was followed by measuring the colony diameter during the incubation period. Only cinnamon leaf, rosemary, thyme, bay and clove essential oils exhibited some antifungal activity against all isolates. The antifungal activity depended on a_w and pH levels. In general, a stronger inhibition was observed as the water availability increased, moreover, in some cases at 0.80 a_w they favoured fungal growth. The interaction between essential oil concentration and pH depended mainly on the essential oil. Rosemary, thyme and bay were more effective at pH 5, loosing their activity as pH increased, while only cinnamon leaf was more effective near neutrality. These findings strengthen the possibility of using plant essential oils as an alternative to chemicals to preserve bakery products.

Key Words: antifungal activity, essential oils, bakery products, moulds, *Eurotium*, *Aspergillus*, *Penicillium*

INTRODUCTION

Products of intermediate moisture and slightly basic pH, cakes are oversensitive to xerophilic mycobiota spoilage (Seiler, 1988; Beuchat and Hocking, 1990; Abellana et al., 1997a; Pitt and Hocking, 1997; Fustier et al., 1998). In cake production, the most common type of microbial spoilage is mould growth and in many cases it is the major factor governing shelf life (Earle and Putt, 1984). The most widespread and probably most important moulds, in terms of biodeterioration of bakery products, are species of *Eurotium*, *Aspergillus* and *Penicillium* (Abellana et al., 1997b). The main variables to be taken into account in predictive microbiology for bakery products design are a_w and pH, as well as atmosphere composition in the package, concentration of preservatives and storage temperature (Marín et al., 2002).

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Since industrialisation, urbanisation and change of life style started to put demands for longer shelf life on bakery products, the use of sorbate, benzoate and propionate has increased (Earle and Putt, 1984). However, today consumers demand less use of synthetic preservatives but still expect food to be free from microbial growth, toxins and other quality deterioration factors (Nielsen and Rios, 2000). Legislation has also restricted the levels and use of some currently accepted preservatives in different foods (Brul and Coote, 1999). The problem for the food industry is to fulfil the demands of minimum changes in food quality and maximum security (Nielsen and Rios, 2000). For this reason, there is a growing interest in the studies of natural additives as potential preservatives (Milos et al., 2000). In this sense, many investigations have been led to find novel natural compounds with preserving activity, capable of substituting the traditional ones (Farag et al., 1989; Mishra and Dubey, 1994; Pattnaik et al., 1996; Hammer et al., 1999; Chao and Young, 2000; Inouye et al., 2000; Hsieh et al., 2001).

Researchers over the last 100 years have demonstrated the antimicrobial properties of several common spice oils (Bullerman et al., 1977). Montes-Belmont and Carvajal (1999) detected activity against *Aspergillus flavus* growth in 36 aqueous and hexane extracts from 107 different plant species. Chao and Young (2000) reported that coriander, cinnamon bark,

lemongrass, savory and rosewood oils were inhibitory for *A. niger*. More than 280 plant species have been investigated for their effect on toxigenic *Aspergillus* spp., and 100 of them have been demonstrated to have some activity on growth or toxin production by these fungi (Montes-Belmont and Carvajal, 1998). Different researchers have checked the antifungal properties of cinnamon and clove essential oils (Bullerman et al., 1977; Salmeron and Pozo, 1991; Patkar et al., 1993; Sinha et al., 1993). The essential oil of oregano inhibited completely the mycelial growth of *A. niger*, *A. flavus* and *A. ochraceus*. However, thyme oil inhibited *A. flavus* and *A. niger* mycelial growth but not that of *A. ochraceus* (Paster et al., 1990).

Despite the high number of publications that documented the antimicrobial activity of essential oils against different fungal species, few reports have dealt with *Eurotium* spp. growth. Since their antimicrobial effects, spices and herbs essential oils are of interest regarding their possible use as alternatives to food preservatives currently in use. The present study aimed to carry out an *in vitro* screening of a range of 20 essential oils for the inhibition of mycelial growth of different species isolated from bakery products (including *Eurotium* spp., *Aspergillus* spp. and *Penicillium* spp.) at different a_w and pH conditions.

MATERIAL AND METHODS

Microorganisms and Essential Oils

Fungal Isolates

A total of 7 isolates from different bakery products were used. Five of them, *Eurotium amstelodami* (3.205), *Eurotium herbariorum* (3.209), *Eurotium rubrum* (3.228), *Aspergillus flavus* (3.226) and *Aspergillus niger* (3.227) were isolated by Abellana et al. (1997b) from Spanish bakery products. The numbers in parentheses are the references numbers for the cultures held at the Food Technology Department, University of Lleida, Spain. The other two isolates, *Eurotium repens* (IBT18000) and *Penicillium corylophilum* (IBT6978) were kindly provided by the Department of Biotechnology of the Technical University of Denmark and had been isolated from Danish bakery products.

Essential Oils

The essential oils (purchased from F.D. Copelans & Sons, Ltd, London) used in this study were extracted from: lemon, aniseed, mandarin, grapefruit, cinnamon leaf, orange, lime, lemongrass, eucalyptus, spearmint, rosemary, thyme, basil, sweet fennel, pine sylvestris, peppermint, ginger, bay, clove and sage.

Methods

Experimental Design

The basic concept of experimental design is to devise a small set of experiments in which all pertinent factors are varied systematically. An important aspect is that they provide mathematical frameworks for changing all pertinent factors simultaneously, and that they achieve this in a small number of experimental runs (Haasum and Nielsen, 1998).

In order to assay the highest number of possible essential oils for their antifungal activity, a screening design was made using a statistical program, MODDE version 5.0 (Umetrics AB, Umeå, Sweden). Two different sets of 10 essential oils were designed separately, as the program did not allow more than 10 levels of qualitative factors at a time. A D-optimal quadratic design with three centre points was used. This is a computer generated design which maximises the information in the entire experimental space with a minimum number of runs. The design resulted in 51 runs for each set of 10 essential oils.

The factors assayed were different essential oils as qualitative factors and a_w (0.80–0.90), pH (5–7.5) and concentrations (0–1,000 ppm) as quantitative factors. The response recorded was colony diameter.

Media Preparation

The basic medium used was a 2% wheat flour agar. It was adjusted to achieve the desired a_w and pH levels as was previously described by Marín et al. (2002). Ten mL of suitable solutions of essential oil (25 and 12.5 mg essential oil/mL for treatments of 1,000 and 500 ppm, respectively, made in water with 0.005% tween 80) per 250 mL of medium were added before autoclaving (121 °C, 98 kPa, 15 min). Ten mL of water with 0.005% tween 80 per 250 mL of medium were added to the control treatments. In this way, the addition of the essential oils before the baking process was simulated. Sterile media were poured into 9 cm-diameter sterile Petri plates. The final a_w and pH of the media were checked after autoclaving and plating with an AquaLab (Decagon, Pullman) and a Crison micropH2000 pH-meter (Crison, Barcelona), respectively.

Inoculation, Incubation and Measurement

For each fungal isolate, a conidial spore suspension of 10^6 spores/mL was prepared and Petri plates were needle-inoculated in the centre. After that, plates were incubated at 25 °C in sealed polyethylene bags in order to maintain a constant relative humidity value (ERH) for 42 days. Diameters of the growing colonies were measured weekly with the aid of a binocular magnifier.

Statistical Treatment of the Results

Results were analysed using The Unscrambler® version 7.6 (CAMO ASA, Oslo, Norway) program. Multivariate analyses of the whole set of responses was done by Partial Least Square (PLS) regression, in order to show correlations between factors and responses.

RESULTS

A partial least squares (PLS) regression method was used for data evaluation. This is a bilinear modelling method that uses simultaneously X- and Y-data matrices to find the best regression model to predict Y-variables. The X-variables (factors) involved in the regression were four quantitative factors (a_w, pH, concentration and time) and the 20 essential oils as binary variables; the seven Y-variables (responses) corresponded to the colony diameters of each of the seven isolates tested. All variables were standardised (1/StDev) prior to the regression analysis to give all variables the same variance, and so the same opportunities to influencing the data analysis.

The model obtained explained 71% of X-variance and 69% of Y-variance in the four first components. The relationships between X- and Y-variables can be studied by interpreting the scatter plot of X-loadings weight and Y-loadings for two specific components. The loading plot for components 1 and 2 (Figure 1a) was the most representative since these two components accounted for most of the variability in the data that can be explained by the model. Responses located close to each other in that plot responded in a similar way to changes in the tested factors. Responses were all correlated to some extent, *A. niger* and *P. corylophilum* having a slightly different behaviour. The farther a factor is located from the origin the more significant the effect it has in the model. If it is projected in roughly the same direction from the centre as a response, it is positively linked to that response and if it is projected in the opposite direction, it is negatively linked. Water activity, time and to a lesser extent pH, were the most important factors describing growth. Some of the essential oils had a negative correlation with growth, while others had no correlation or positive correlation, then, as the variable "concentration" included all the essential oils together, its mean effect was almost negligible. Finally, all essential oils had little effect if compared with that of the other factors. Component 4 explained the effect of essential oils on growth (Figure 1b). However, it has to be noted that only the 1.7% of the total variance in the data was explained by this component.

The most important essential oil inhibiting fungal growth was cinnamon leaf as it had a negative correlation to all species growth. Thyme, bay, rosemary, clove, lemongrass and ginger essential oils exhibited also a negative correlation with fungal responses, but to a lesser extent. The regression coefficients of the model equation also express the link between different factors and different variables but taking into account all useful components together (Table 1). Cinnamon leaf, rosemary, thyme, bay and clove were those essential oils with potential antifungal capacity against all species tested, while lemongrass and ginger were effective only for some species. Finally aniseed and sage, among others, significantly stimulated fungal growth.

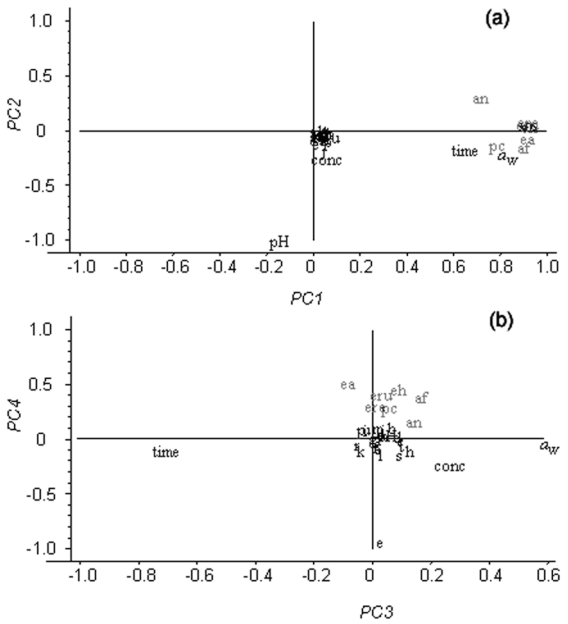

Figure 1. Loadings plot of the PLS regression showing the relationship of water activity (a_w), pH, concentration (conc.), essential oils (a = lemon, b = aniseed, c = mandarin, d = grapefruit, e = cinnamon leaf, f = orange, g = lime, h = lemongrass, i = eucalyptus, j = spearmint, k = rosemary, l = thyme, m = basil, n = sweet fennel, o = pine sylvestris, p = peppermint, r = ginger, s = bay, t = clove and u = sage) and time on fungal growth (ea = *E. amstelodami*, eh = *E. herbariorum*, ere = *E. repens*, eru = *E. rubrum*, an = *A. niger*, af = *A. flavus* and p = *P. corilophylum*) after 28 days of incubation. (a) First and second principal components (*PC1*, *PC2*), (b) third and fourth principal components (*PC3*, *PC4*).

In a second step, PLS regression was performed individually for each of the five essential oils that had shown to be more effective. a_w*concentration and pH*concentration interactions were added to lineal terms. In general, for all five analysis, the most important single factors affecting colony diameters were a_w and time. Concentration, pH and both interactions effects were different depending on the essential oil, so

Table 1. Regression coefficients for the X-variables involved in the model obtained by PLS regression.

Variable	*E. amstelodami*	*E. herbariorum*	*E. repens*	*E. rubrum*	*A. niger*	*A. flavus*	*P. corylophilum*
a_w	+0.63	+0.72	+0.64	+0.66	+0.54	+0.76	+0.60
pH	−0.15	−0.26	−0.30	−0.27	−0.49	−0.09	−0.07
Lemon	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Aniseed	+0.11	+0.11	+0.08	+0.10	+0.07	+0.10	+0.08
Mandarin	+0.01	+0.03	+0.02	+0.02	+0.02	+0.02	+0.01
Grapefruit	+0.04	+0.05	+0.03	+0.04	+0.04	+0.04	+0.02
Cinnamon leaf	−0.52	−0.47	−0.34	−0.43	−0.23	−0.40	−0.32
Orange	0.00	0.00	0.00	0.00	0.00	−0.01	0.00
Lime	0.00	0.00	0.00	0.00	−0.02	0.00	0.00
Lemongrass	−0.04	−0.02	−0.01	−0.02	+0.02	−0.01	−0.02
Eucalyptus	+0.06	+0.04	+0.02	+0.04	0.00	+0.03	+0.02
Spearmint	+0.09	+0.07	+0.04	+0.06	0.00	+0.08	+0.07
Rosemary	−0.04	−0.05	−0.04	−0.04	−0.03	−0.05	−0.04
Thyme	−0.04	−0.04	−0.03	−0.04	−0.02	−0.03	−0.03
Basil	+0.09	+0.08	+0.06	+0.07	+0.04	+0.06	+0.05
Sweet fennel	+0.05	+0.05	+0.04	+0.05	+0.03	+0.04	+0.03
Pine sylvestris	+0.07	+0.07	+0.05	+0.06	+0.04	+0.05	+0.04
Peppermint	+0.11	+0.08	+0.06	+0.08	+0.02	+0.07	+0.07
Ginger	+0.02	0.00	+0.08	0.00	−0.01	0.00	0.00
Bay	−0.08	−0.07	−0.06	−0.06	−0.03	−0.06	−0.05
Clove	−0.04	−0.03	−0.03	−0.03	−0.09	−0.02	−0.03
Sage	+0.14	+0.12	+0.10	+0.12	+0.06	+0.11	+0.09
Concentration	−0.16	−0.13	−0.13	−0.14	−0.10	−0.08	−0.08
Time	+0.57	+0.44	+0.49	+0.48	+0.27	+0.38	+0.40

they will be described separately. Unless otherwise specified, all seven species responses were positively correlated; this means that factors influenced their growth in a similar way.

Cinnamon Leaf Essential Oil

A three component model was developed with an explained calibration variance (ECV) of 65.2% and with an explained validation variance (EVV) of 63.2%. a_w*concentration was the most important interaction affecting fungal growth. Contour plots display response values as contour lines, so that the response value for any combination of levels of the designed variables can be easily estimated. The response surfaces of *E. herbariorum* growth at both pH levels tested are displayed in Figure 2. Cinnamon leaf oil was more effective at pH 7.5 than at pH 5. At 0.90 a_w there was a marked inhibition of the growth with increasing concentration values, while at 0.80 and 0.85 a_w there was no effect or even a stimulation of growth. Even though the general effect of cinnamon leaf essential oil in *A. niger* growth was the same, it was less sensitive to increasing concentrations of the oil (data not shown).

Rosemary Essential Oil

A four component model was developed (ECV = 68.5%; EVV = 67%). In this case, the interaction a_w*concentration was not so significant, while pH*concentration became more important, making the essential oil quite active at pH 5 and loosing the activity at the higher pH level (Figure 3). Moreover, at pH 7.5 and 0.80 a_w, increasing concentrations seemed to enhance fungal growth.

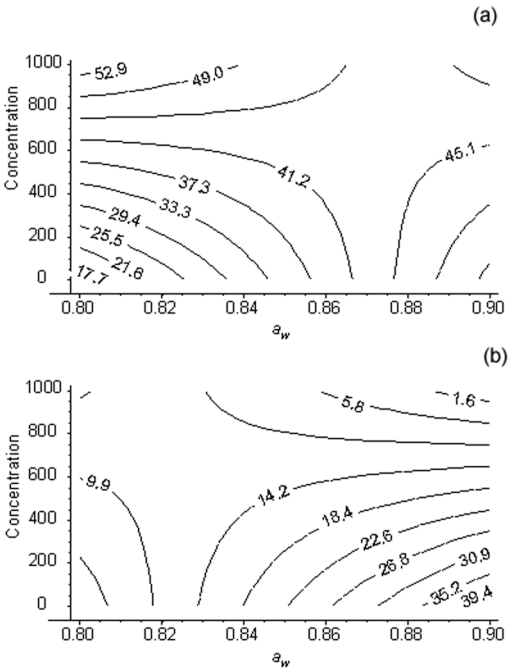

Figure 2. Contour plots based on PLS regression showing *E. herbariorum* growth (mm of colony diameter) as affected by cinnamon leaf essential oil (ppm) and a_w levels after 28 days of incubation. (a) pH 5, (b) pH 7.5.

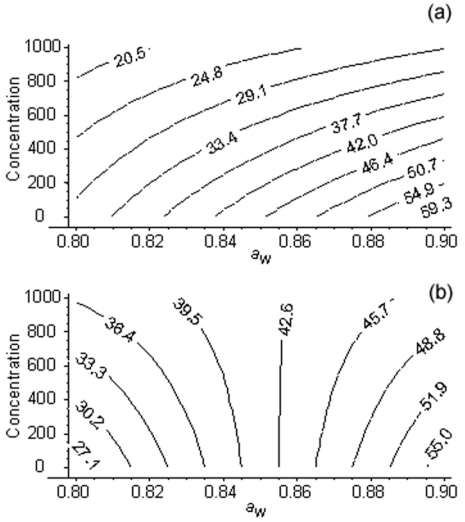

Figure 3. Contour plots based on PLS regression showing *E. amstelodami* growth (mm of colony diameter) as affected by rosemary essential oil (ppm) and a_w levels after 28 days of incubation. (a) pH 5, (b) pH 7.5.

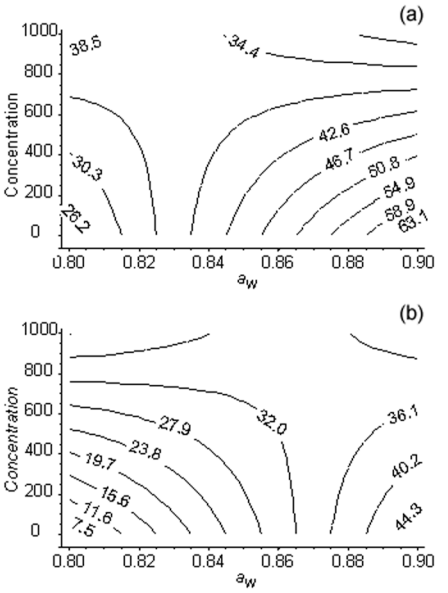

Figure 4. Contour plots based on PLS regression showing *E. repens* growth (mm of colony diameter) as affected by thyme essential oil (ppm) and a_w levels after 28 days of incubation. (a) pH 5, (b) pH 7.5.

Thyme Essential Oil

A three component model was developed (ECV = 68.7%; EVV = 67.8%). Only at high a_w (0.85–0.90), colony diameters could be reduced by increasing concentrations of essential oil (Figure 4). Comparing both pH levels, this essential oil was practically ineffective near to neutrality. In this case, *A. niger* presented the same behaviour but was more sensitive at pH 7.5 (data not shown).

Bay Essential Oil

A four component model was developed (ECV = 69.2%; EVV = 68%). Colony diameters decreased with the increasing concentration of essential oil, regardless of a_w levels (Figure 5). Antifungal effect was more pronounced at the lower pH. *A. niger* growth was unaffected by this essential oil addition at pH 7.5 regardless of a_w (data not shown).

Clove Essential Oil

A four component model was developed (ECV = 69.7%; EVV = 68.5%). a_w*concentration was the main interaction. In general, at both pH levels and at 0.90 a_w, the essential oil was more effective in inhibiting fungal growth than at 0.80–0.85 a_w (Figure 6). *E. amstelodami* was an exception, because its growth depended more on pH level than on a_w level, being more inhibited at pH 7.5 in all a_w range (Figure 7).

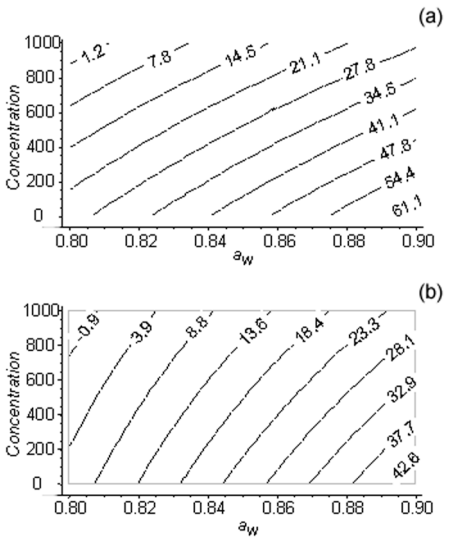

Figure 5. Contour plots based on PLS regression showing *E. repens* growth (mm of colony diameter) as affected by bay essential oil (ppm) and a_w levels after 28 days of incubation. (a) pH 5, (b) pH 7.5.

In summary, all five essential oils were only effective, or at least had more antifungal effect, at high a_w. Moreover, in some cases at 0.80 a_w they enhanced fungal growth. The interaction between essential oil effectiveness and pH was more complex, and depended mainly on the essential oil being assayed: rosemary, thyme and bay were more effective at pH 5, loosing their activity as pH increased. Cinnamon leaf

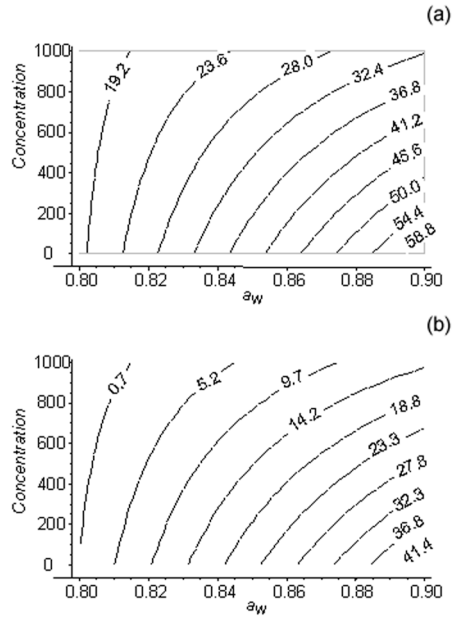

Figure 6. Contour plots based on PLS regression showing *E. herbariorum* growth (mm of colony diameter) as affected by clove essential oil (ppm) and a_w levels after 28 days of incubation. (a) pH 5, (b) pH 7.5.

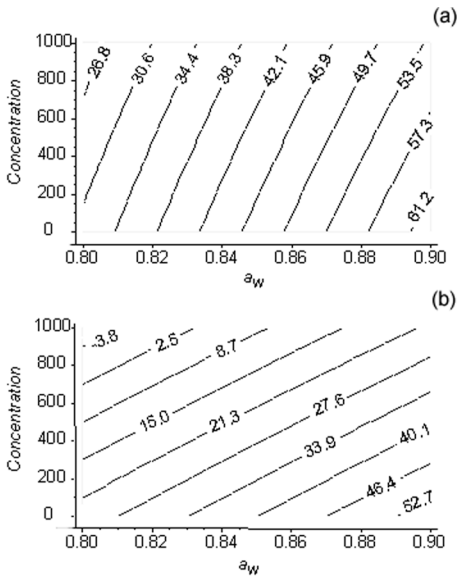

Figure 7. Contour plots based on PLS regression showing *E. amstelodami* growth (mm of colony diameter) as affected by clove essential oil (ppm) and a_w levels after 28 days of incubation. (a) pH 5, (b) pH 7.5.

was the only one which was more effective near neutrality and finally clove essential oil exhibited similar antifungal activity at both pH levels tested.

DISCUSSION

Almost all species tested were affected in a similar way by the five essential oils detected as having the strongest antifungal activity. Among the remaining essential oils, only lemongrass and ginger, influenced fungal growth in a different way depending on the isolates. *A. niger* growth was enhanced by lemongrass addition, however it was the only species inhibited to some extent by ginger essential oil, while growth of the remaining species was favoured or not affected by this essential oil. By contrast, other authors have shown that *A. niger* is greatly inhibited by lemongrass essential oil, and not affected by ginger essential oil (Chao and Young, 2000).

Different methodologies have been used to test the antifungal activity of these compounds, therefore comparison between results becomes difficult (Delaquis and Mazza, 1995). The disk diffusion technique, the agar dilution and the broth dilution methods are the most commonly used. The results obtained by these methods may differ, as many factors may vary, such as the solubility of the oil or oil components, the use and concentration of emulsifiers and the exposure of the microorganism to the oil (Hammer et al., 1999). In the present study essential oils were added to the medium, so only the soluble portion of the oil has been tested, being the volatile portion contribution probably obviated. Moreover, the composition of plant oil extracts varies according to agronomic conditions and harvest time making the comparison between different studies more difficult (Mishra and Dubey, 1994; Guillén and Cabo, 1996).

To better simulate the baking process, essential oils were added before autoclaving the medium, being exposed to high temperatures (15 min at 121 °C). This could be the reason why some of the essential oils which were expected to have antifungal activity (based on literature) failed to control fungal growth, although some researchers have reported the heat stability of some essential oils (Hsieh et al., 2001; Hsieh, 2000). The compounds responsible for the antimicrobial activity probably suffer some chemical reaction or evaporate.

Contrary to the combined hurdle theory, all five essential oils were more effective in controlling fungal growth at high a_w level, loosing activity as a_w decreased. Although antifungal activity of almost all essential oils was found to depend on pH levels, no clear relationship was found between activity and medium acidity. More knowledge about the mechanism by which essential oils act inhibiting fungal growth is necessary to understand the relation with pH and water availability. In this sense, many researches have led to the isolation

of the main components of essential oils exhibiting antimicrobial activity (Farag et al., 1989; Montes-Belmont and Carvajal, 1998; Del Campo et al., 2000; Arras and Usai, 2001; Vázquez et al., 2001; Mahmound, 1994; Ultee et al., 2002). It appears that there is a relationship between the chemical structures of the most abundant compounds in the essential oils and the antifungal effect (Farag et al., 1989). Generally, the extent of the inhibition of the oils can be attributed to the presence of an aromatic nucleus containing a polar functional group. However other factors such as the hydrophilic/lipophilic balance are likely to be involved; for example, the phenolic-OH groups that are very reactive and can easily form hydrogen bonds with active sites of enzymes (Farag et al., 1989). Chang et al. (2001) looking for the specific chemical structure responsible for the strong antimicrobial activity of cinnamaldehyde (main component of cinnamon essential oil) concluded that a conjugated double bond and a long CH chain outside the ring was responsible for the main antibacterial activity. Carvacrol (main component of thyme essential oil), a phenolic compound, may penetrate the cytoplasm membrane causing a destabilisation and in addition could act as a proton exchanger reducing the pH gradient across the membrane (Ultee et al., 2002). Del Campo et al. (2000) found out that the most apolar phenolic compounds (phenolic diterpenoids) were presumably responsible for the antimicrobial activity of a rosemary extract.

After this preliminary study, more investigations must be carried out to ensure the suitability of applying cinnamon leaf, rosemary, thyme, bay and clove essential oils to control bakery products spoilage. An important aspect to prevent is the strong flavour of these compounds that could modify the sensory characteristic of foods. On the other hand, some researchers have pointed out the lack of effectiveness of essential oils when they are applied in foods, as a consequence of reactions with lipids and proteins (Del Campo et al., 2000; Davidson and Parish, 1989).

ACKNOWLEDGEMENTS

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