Original article

The effect of temperature on microbial growth in apple cubes packed in film and preserved by use of orange juice

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Summary Red delicious apple cubes were packed in fresh orange juice containing chemical preservatives (citric and ascorbic acid, potassium sorbate) and covered with plastic films of different gas permeabilities (polyethylene and EVA-SARAN-EVA) before storage at 4, 10 and 20 °C. The concentration of potassium sorbate in the product was optimized with respect to colour and microbial growth. Yeast and mould growth was modelled by Gompertz and linear equations to derive parameters such as lag phase, maximum microbial population and specific microbial growth or rates of decline. Activation energies for the specific growth rates were estimated from Arrhenius-type equations and the time required to reach microbial counts of $10^{6\pm0.2}$ CFU g^{-1} was determined in all cases. At 4 °C, these values were longer than 25 days in all systems tested. The use of a low gas permeability film and an adequate potassium sorbate concentration extended storage life at higher temperatures.

Keywords Chemical preservatives, modelling microbial growth, packaging film, refrigeration, storage.

Introduction

'Ready-to-eat' fruit salads are prepared by using fresh fruits and low pH fruit juices such as that from oranges. Cut apples are the common ingredient. Processing, although minimal, may increase microbial spoilage in the fruit by transferring skin microflora to fruit flesh where micro-organisms can grow rapidly (Pao *et al*., 1997, 1998; Buta *et al*., 1999; Sizer & Balasubramaniam, 1999). The low pH of most fruits restricts microflora to acid-tolerant micro-organisms such as fungi and lactic acid bacteria (Parish, 1991; O'Connor-Shaw *et al*., 1994). The National Food Processors Association of USA has established that juices used either as fruit products or ingredients have to be pasteurized, or else must receive an equivalent treatment to ensure the production of juice free from pathogenic micro-organisms (Matthys, 1999; Mermelstein, 1999). The inherent risks accompanying consumption of non-pasteurized products require the use of chemical preservatives such as sorbate or benzoate to inhibit microbial growth (Bizri & Wahem, 1994).

Sorbic acid has known antimicrobial properties in a wide range of products. The salts of sorbic acid, especially the potassium salt, are extensively used because of their high water solubility. From the time of its earliest use, potassium sorbate was classified as relatively non-toxic, being metabolized by organisms in a similar way to naturally occurring fatty acids. The World Health Organisation has established an acceptable daily intake of 25 mg of sorbate per kg of body weight (Sofos & Busta, 1981).

The use of low storage temperatures and low gas permeability films allow shelf-life of vegetable

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products to be extended. The effectiveness of refrigerated storage as a preservation method depends on the initial quality of the raw material and the time-temperature conditions.

In a previous work (Andrés *et al*., 2001, 2002) using several varieties of apple, the stability of fruit systems composed of apple cubes maintained in orange juice containing various chemical preservatives was studied. The results demonstrated that sodium bisulphite could be replaced by citric and ascorbic acids in Red delicious apples.

The objectives of the present work were:

1 to optimize the formulation of a fruit storage system composed of Red delicious apples in fresh Valencia orange juice using natural preservatives such as citric acid and ascorbic acid and thereby minimizing the concentration of potassium sorbate. The optimization was based on the consideration of quality parameters such as colour and growth of moulds and yeast.
2 To analyse the effects of packaging film gas permeability [polyethylene and EVA-SARAN-EVA (ESE)], storage temperatures (4, 10 and 20 °C) and potassium sorbate concentrations on microbial growth in the test system.
3 To model the effect of temperature on microbial growth parameters and to determine product stability.

Materials and methods

Red delicious apples and Valencia oranges were purchased over two consecutive years (2000–2001, March to December) in the local market and stored at 10 °C for 24 h before performing the tests.

To obtain fresh orange juice, instruments, equipment and oranges were washed and brushed manually with sodium hypochlorite solution (0.25 g kg^{-1}) and finally with tap water as described in previous studies (Andrés *et al*., 2001).

Apples were washed in tap water, manually peeled and cut to give 1 cm side cubes. Samples were comprised of 30 g portions of Red delicious cubes in contact with 30 ml of orange juice; this system also contained 1 g citric acid per kg product, 0.150 g ascorbic acid per kg product and 20 g sucrose per kg product. These samples were identified as a system with natural preservatives (SNP) and its formulation was based on previous work (Andrés *et al*., 2002). The storage life of these samples was very short (5 days). To prolong the storage life, potassium sorbate had to be added to these samples. In the first part of the work, the concentrations of potassium sorbate that were tested ranged from 0.125 to 0.500 g per kg product and were selected to comply with values permitted by Argentine regulations (Código Alimentario Argentino, 1969). This standard establishes a maximum level of 1 g of sorbate per kg of product. The reagents used were of analytical grade: ascorbic and citric acids (Cicarelli, Santa Fe, Argentina) and potassium sorbate (Inmobal, Nutrer, Buenos Aires, Argentina). Sucrose (Ledesma, Buenos Aires, Argentina) was used as a sweetener.

To analyse the effect of the gas film permeability, half of the samples were packaged in polyethylene bags (oxygen permeability, $PO_2 =$ 5000 cm^3 m^{-2} day^{-1} atm^{-1} at 23 °C) whereas the other half were vacuum-packaged in ESE film (Grace, Buenos Aires, Argentina) ($PO_2 =$ 50 cm^3 m^{-2} day^{-1} atm^{-1} at 23 °C). Bags were heat sealed.

Samples were stored at 4, 10 and 20 °C and periodically tested for yeast and moulds counts.

Experimental design

The first set of experiments was carried out by storing the samples containing different concentrations of potassium sorbate: 0, 0.125, 0.250 and 0.500 g per kg product, and packaged with polyethylene or ESE packaging films at 10 °C for 24 days. This was performed to determine the minimum concentration of potassium sorbate that must be added to the SNP system to maintain the main quality attributes of the products such as surface colour and microbial growth, at an acceptable level. In the second set of experiments, the effect of storage temperature (4, 10 and 20 °C) on microbial growth was tested for formulations that had been previously optimized. Each set of experiments was run on duplicate samples. The total storage time tested was extended to 25 days in these experiments. Microbial counts at 4, 10 and 20 °C were mathematically modelled as a function of time.

Colour measurements

The surface colour of the apple samples was measured with a tristimulus reflectance colorimeter (Minolta CR-300; Minolta Corp., Ramsey, NJ, USA) on 16 peeled apple cubes, each having sides of 1.5 cm. Colour was recorded by using the CIE-$L^*a^*b^*$ scale, where L^* indicates lightness, a^* indicates chromaticity on a green (−) to red (+) axis, and b^* chromaticity on a blue (−) to yellow (+) axis. Numerical values of the parameters were converted into Δa^* ($\Delta a^* = a^* - a^*_i$), Δb^* ($\Delta b^* = b^*_i - b^*$), ΔL^* ($\Delta L^* = L^*_i - L^*$) and chromaticity difference [chromaticity difference $= (\Delta a^{*2} + \Delta b^{*2})^{1/2}$]. Initial colour was measured on apple cubes after immersion in the corresponding juices for 2 h at 10 °C. The colour of untreated apple cubes (without juice) was also measured at 10 °C. All measurements were in quadruplicate.

Microbial growth

At different storage times, 20 g of sample (apple cubes and juice) were homogenized with 80 ml 0.1% peptone in a Stomacher (Seward Medical, London, UK) for 60 s. Duplicate serial dilutions of Yeast Glucose Chloramphenicol agar (YGC) (Merck Darmstadt, Germany) were plated for the estimation of mould and yeast counts (5 days at 30 °C).

Modelling of microbial growth

The modified Gompertz equation (Gibson & Roberts, 1989; Zwietering *et al.*, 1990) was applied:

$$\log N = a + c \cdot \exp\{-\exp[-b(t-m)]\} \qquad (1)$$

where $\log N$ was the decimal logarithm of microbial counts [$\log(\text{CFU ml}^{-1})$] at time t; a was the $\log N_0$ asymptotic log count as time decreases indefinitely [$\log(\text{CFU ml}^{-1})$]; c was the log count increment as time increases indefinitely [$\log(\text{CFU ml}^{-1})$]; m was the time required to reach the maximum growth rate (days) and b was the relative growth rate at time m (days^{-1}).

From these parameters, the exponential growth rate ($\mu = bc/e$) [$\log(\text{CFU ml}^{-1})\ \text{day}^{-1}$], with $e = 2.7182$, lag phase duration [$\text{LPD} = m - (1/b)$ (days)] and maximum population density, $\text{MPD} = a + c$ [$\log(\text{CFU ml}^{-1})$] were derived. The Gompertz equation was applied to every culture in which microbial growth was detected. The equation was fitted to growth data using non-linear regression routine of the SYSTAT software (SYSTAT, Evanston, IL, USA). The algorithm selected calculated the set of parameters with the lowest residual sum of squares (RSS) and 95% confidence interval.

When preservatives produced a bactericidal effect, a linear model was applied:

$$\log N = \log N_0 + R(t - \text{LPD}) \qquad (2)$$

where R is the decline rate [$\log(\text{CFU ml}^{-1})\ \text{day}^{-1}$]; this had negative values.

Statistical analysis

Analysis of variance (ANOVA) and the test of mean comparisons according to Fisher LSD, were used; levels of significance were 0.05 and 0.01. The statistical computer system package (SYSTAT Inc., version 5.0) was used in all cases.

Results and discussion

Optimization of potassium sorbate concentration in the product stored at 10 °C

The effect of sorbic acid on the growth of moulds and yeasts was analysed by considering that the antimicrobial action of weak acids is generally attributed to their undissociated fraction. Sorbic acid is a monoprotic weak acid ($pK_a = 4.75$). The undissociated concentration of a weak acid (uac) depends on the total concentration and pH:

$$\text{uac} = C_t[H^+]/(K_a + [H^+]) \qquad (3)$$

Table 1 Total (C_t) and undissociated sorbic acid concentration (uac) as a function of potassium sorbate in the tested formulations

Potassium sorbate (g per kg product)	pH	C_t (mM) in the juice	uac (mM) in the juice
0	3.57	0	0
0.125	3.62	1.66	0.089
0.250	3.70	3.33	0.165
0.500	3.55	6.66	0.380

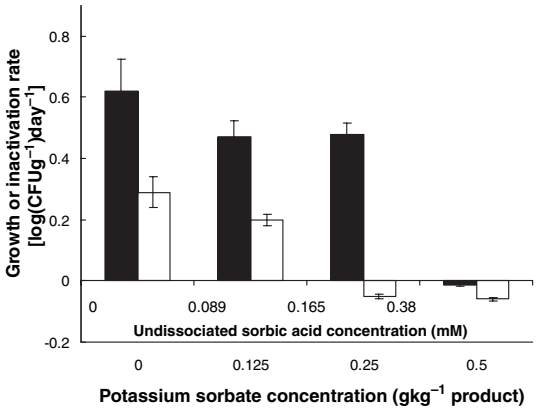

Figure 1 Effect of potassium sorbate concentration on the growth or inactivation rates of moulds and yeast in Red delicious apple cubes at 10 °C in ■ polyethylene and □ ESE films. Bars indicated standard error of the mean.

where C_t is the total acid concentration and K_a is the dissociation constant of sorbic acid.

Table 1 lists the pH of orange juice as well as total and undissociated sorbic acid concentrations (uac). Figure 1 shows the effect of potassium sorbate concentration on the growth or inactivation rate of moulds and yeasts in apple cubes immersed in the orange juice systems during storage at 10 °C. The addition of different potassium sorbate concentrations modified the parameters of the Gompertz and linear equations.

In polyethylene, samples containing 0.25 g potassium sorbate per kg product (pH = 3.70, uac = 0.165 mM) exhibited microbial growth, although μ values decreased by 25% compared with the control. Using this film, 0.500 g potassium sorbate per kg product (pH = 3.55, uac = 0.38 mM) gave a microbicidal effect. For LPD values ranging from 2.8 to 3.5 days, and MPD values between 7.8 and 6.8 log CFU g^{-1}, no effect of potassium sorbate concentration ($P < 0.05$) was found.

In ESE film, for potassium sorbate concentrations ranging between 0 and 0.125 g per kg product, μ values were half those measured when using polyethylene (Fig. 1). With 0.125 g potassium sorbate per kg of product (pH = 3.62, uac = 0.089 mM) and with ESE film, LPD was double whereas MPD was half the corresponding control values.

A microbicidal effect was apparent at concentrations higher than 0.125 g potassium sorbate per

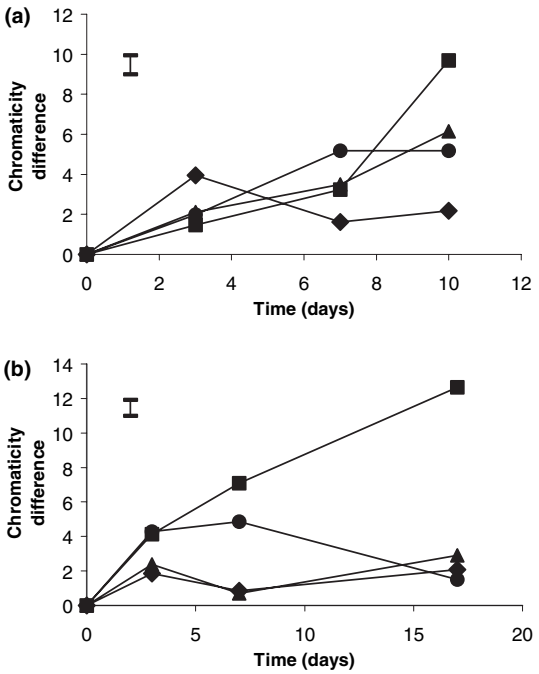

Figure 2 Chromaticity difference values as a function of storage time at 10 °C of apple cubes immersed in system added with natural preservatives (SNP) and in systems added with natural preservatives plus potassium sorbate at different levels (SNP + KS). Bars indicate least significant difference, LSD, ($P < 0.05$). (a) polyethylene, (b) ESE film. (◆) SNP; (●) SNP + 0.125 g KS per kg product; (▲) SNP + 0.250 g KS per kg product; (■) SNP + 0.500 g KS per kg product.

kg of product; however, at these concentrations, apples experienced severe browning with large modification of chromaticity difference values (>6) (Fig. 2a,b). These values were lower for ESE than for polyethylene packaging ($P < 0.01$) showing that, as expected, films with low oxygen permeability lead to a better colour retention in apples (Fig. 2).

In levels ranging from 0 to 0.125 g kg^{-1}, potassium sorbate permitted good colour retention (chromaticity difference <6) (Fig. 2).

Therefore, the best concentration of potassium sorbate for the preservation of Red delicious apple cubes was 0.125 g kg^{-1} product (0.089 mM uac) with citric and ascorbic acids. At this level, potassium sorbate showed an inhibitory effect on moulds and yeasts in vacuum packaged samples and maintained an adequate colour. This optimized formulation was identified as SNP + KS.

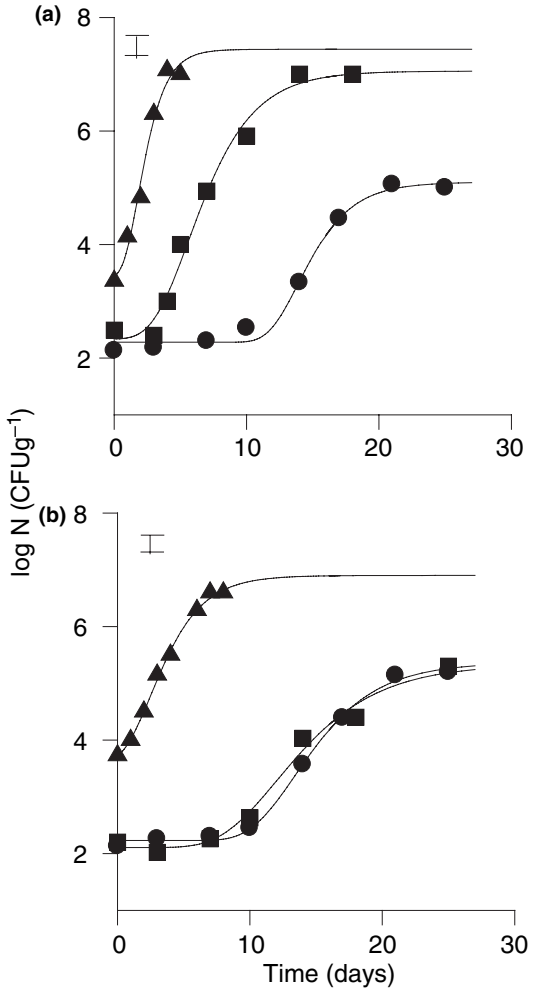

Figure 3 Mould and yeast counts in apple cubes immersed in the system added with natural preservatives (SNP) as a function of time at different temperatures. Solid lines correspond to Gompertz mathematical models fitted to the experimental data at the various conditions. Bars indicate least significant difference, LSD, ($P < 0.05$). (a) polyethylene, (b) ESE film. (●) 4 °C; (■) 10 °C; (▲) 20 °C.

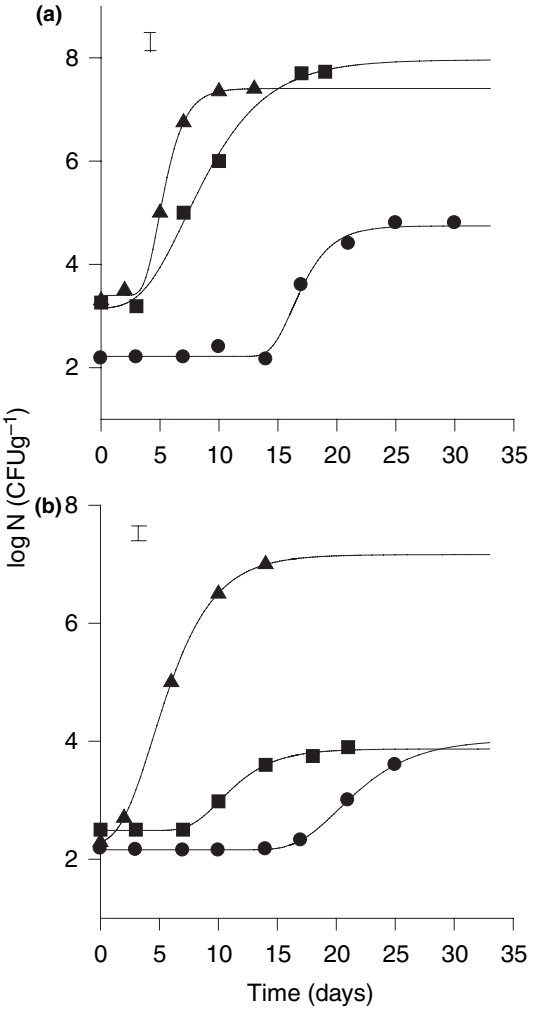

Figure 4 Mould and yeast counts in apple cubes immersed in a system added with natural preservatives plus 0.125 g of potassium sorbate per kg product (SNP + KS) as a function of time at different temperatures. Solid lines correspond to Gompertz mathematical models fitted to data from the several conditions tested. Bars indicate least significant difference, LSD, ($P < 0.05$). (a) polyethylene, (b) ESE film. (●) 4 °C; (■) 10 °C; (▲) 20 °C.

Effect of storage temperature (4, 10 and 20 °C) on moulds and yeasts in the selected formulation

Once the formulation was optimized, the growth of moulds and yeasts was analysed at 4 and 20 °C and results were compared with data at 10 °C. Figure 3 shows microbial counts at 4, 10 and 20 °C in Red delicious apple cubes treated with natural preservatives (SNP) as well as curves predicted by the Gompertz model. Figure 3a shows samples packaged in polyethylene film and Fig. 3b shows those in ESE film. Figure 4a,b shows microbial growth on apple cubes in the SNP + KS optimized formulation at the same temperatures. Gompertz fitting and derived parameters are summarized in Table 2.

In polyethylene, as storage temperature increased from 4 to 20 °C, the exponential growth rate (μ) increased 2.5 to 3 times [0.47–1.27 log(CFU g^{-1})

Table 2 Effect of storage temperature (4, 10 and 20 °C) and packaging film (polyethylene or ESE) on Gompertz and derived parameters obtained from yeast and mould counts in Red delicious apple cubes, immersed in orange juice added with natural preservatives (SNP), or SNP plus potassium sorbate (SNP + KS)

			Gompertz model						
			Parameters				Derived parameters		
Packaging film	Treatment	T^a(C)	*a* [log (CFU g^{-1})]	*b* (day^{-1})	*c* [log (CFU g^{-1})]	*m* (days)	μ [log (CFU g^{-1}) day^{-1}]	LPD (days)	MPD [log (CFU g^{-1})]
Polyethylene	SNP	4	2.28 (0.07)	0.46 (0.13)	2.79 (0.16)	13.9 (0.37)	0.47 (0.07)	11.78 (0.72)	5.07 (0.17)
		10	2.33 (0.27)	0.36 (0.07)	4.72 (0.20)	5.68 (0.35)	0.62 (0.07)	2.84 (0.64)	7.08 (0.34)
		20	3.44 (0.41)	0.86 (0.09)	4.02 (0.12)	1.84 (0.33)	1.27 (0.10)	0.67 (0.35)	7.47 (0.43)
	SNP + KS	4	2.21 (0.06)	0.55 (0.17)	2.53 (0.12)	16.24 (0.34)	0.52 (0.07)	14.42 (0.66)	4.74 (0.13)
		10	3.15 (0.27)	0.26 (0.14)	4.81 (0.10)	7.19 (0.63)	0.47 (0.03)	3.44 (2.16)	7.97 (0.29)
		20	3.39 (0.33)	0.31 (0.23)	4.00 (0.16)	4.89 (0.90)	1.20 (0.05)	1.66 (2.56)	7.39 (0.37)
ESE	SNP	4	2.23 (0.05)	0.29 (0.03)	3.13 (0.13)	13.45 (0.27)	0.33 (0.04)	10.00 (0.45)	5.36 (0.14)
		10	3.34 (0.23)	0.22 (0.05)	3.67 (0.21)	7.57 (0.90)	0.29 (0.05)	3.05 (1.35)	7.07 (0.31)
		20	3.58 (0.01)	0.46 (0.02)	3.32 (0.02)	2.53 (0.62)	0.62 (0.01)	0.65 (0.63)	6.90 (0.02)
	SNP + KS	4	2.16 (0.01)	0.28 (0.02)	1.86 (0.06)	20.20 (0.15)	0.19 (0.02)	16.6 (0.30)	4.02 (0.06)
		10	2.48 (0.03)	0.39 (0.06)	1.38 (0.06)	10.15 (0.27)	0.21 (0.02)	7.60 (0.48)	3.85 (0.07)
		20	2.24 (0.01)	0.35 (0.05)	4.91 (0.03)	4.46 (0.03)	0.63 (0.01)	1.61 (0.41)	7.16 (0.03)

Gompertz parameters are *a*, *b*, *c* and *m*, being the derived parameters the specific growth rate (μ), lag phase duration (LPD) and maximum population density (MPD). Standard error are given within parenthesis.
SNP = 1 g citric acid/kg of product + 0.150 g ascorbic acid per kg product + 20 g sucrose per kg product.
SNP + KS = SNP + 0.125 g potassium sorbate per kg product.

days] for SNP. In the SNP + KS system, μ values were 0.52–1.20 log(CFU g^{-1}) day^{-1}. At the temperatures tested, potassium sorbate did not show a significant ($P > 0.05$) effect on μ, LPD or MPD.

In low oxygen permeability film (ESE packaging), SNP and SNP + KS samples showed lower μ values than in polyethylene for all temperatures tested. Increasing the temperature from 4 to 20 °C caused a 10-fold decrease in LPD for the tested systems (Figs 3 and 4, Table 2). LPD values obtained for SNP + KS were double those for SNP. Values of MPD decreased from 5.36 to 4.02 at 4 °C and from 7.07 to 3.85 log(CFU/g) at 10 °C because of potassium sorbate addition. Thus, in ESE film, MPD and μ values were more influenced by storage temperature than by potassium sorbate concentration.

The effect of storage temperature on the exponential growth rate (μ) of micro-organisms growing in the formulations tested was determined using the Arrhenius's equation:

$$\mu = A \exp(-E_\mu/RT) \qquad (4)$$

where μ is the exponential growth rate [log (CFU g^{-1}) day^{-1}], T is the absolute temperature (K), E_μ is the activation energy (KJ mol^{-1}), A is the pre-exponential factor [log(CFU/g) $days^{-1}$] and R, the gas constant (8.31 K J mol^{-1}).

Arrhenius activation energies of μ (Table 3) for mould and yeast growth were calculated. The activation energy of a micro-organism represents the sensitivity of growth rate to temperature changes. E_μ values were observed to be highest for SNP + KS samples packaged in ESE film (Table 3).

Table 3 Application of Arrhenius equation to evaluate temperature effect on the specific growth rate of moulds and yeast growing in Red delicious cubes immersed in the natural preservative system (SNP), and in SNP added with 0.125 g potassium sorbate per kg product (SNP + KS). Samples were packaged in polyethylene or ESE films

	Arrhenius equation		
Packaging film	Ln A log (CFU g^{-1}) day^{-1}	E_μKJ mol^{-1}	R^2
SNP in polyethylene	17.69	42.58	0.98
SNP in ESE film	1.29	28.89	0.73
SNP + KS in polyethylene	15.63	37.88	0.77
SNP + KS in ESE film	21.24	53.09	0.87

Ln A, (CFU g^{-1}) day^{-1}; E_μ, activation energy; R^2, coefficients of determination.

Table 4 Effect of temperature, packaging film and potassium sorbate on microbial stability (time to reach $10^{6\pm0.2}$ CFU g^{-1}) of Red delicious cubes immersed in the natural preservative system (SNP), and in SNP added with 0.125 g potassium sorbate per kg product (SNP + KS)

		Stability (days)	
Formulation	**Temperature (°C)**	**Polyethylene**	**ESE film**
SNP	4	>25	>25
	10	9	13
	20	3.2	5
SNP + KS	4	>25	>25
	10	9.5	>25
	20	6	8.3

Microbial stability of the product was defined as the time required for the mould and yeast counts to reach a value of 6 ± 0.2 log CFU g^{-1} with the absence of pathogen micro-organisms (Brackett, 1994; Andrés *et al*., 2001). Confidence limits for microbial stability were calculated by considering an average experimental error in the microbial counts of 0.2 log CFU g^{-1}.

Table 4 shows the stability of the systems with respect to microbial growth that was obtained with natural preservatives (SNP) and an SNP + KS formulation containing 0.125 g potassium sorbate per kg product (uac = 0.089 mM), packaged in different plastic films and stored at 4, 10 and 20 °C.

At 4 °C, the microbial stability for all systems tested and all packaging conditions was more than 25 days. At 10 °C, potassium sorbate addition was required to prolong the stability period. In this regard, in samples packaged in ESE film receiving a potassium sorbate concentration of 0.125 g/kg, the stability period was more than 25 days; this value was 2.6 times as long as the period obtained in polyethylene (9.5 days).

The combination of potassium sorbate and low gas permeability film allowed microbial stability at 10 and 20 °C to be extended to 25 and 8.3 days, respectively.

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