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Short communication

Characterisation of sorbate geometrical isomers

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Abstract

trans,trans Isomers of sorbic acid, its potassium salt and ethyl sorbate isomerise under UV irradiation. All four geometrical isomers of the acid, salt and ester were separated using high-performance liquid chromatography on a nonpolar reversed-phase column (C_{18}) and the ester also by gas chromatography on a VOCOL capillary column. The limit of detection and the interval of linearity were determined for all chromatographic methods. Individual isomers were identified with NMR analysis. Resolved chemical shifts of protons adjacent to the double bonds enabled qualitative and quantitative determination of isomers in the mixture. Antimicrobial activity of potassium sorbate isomers was tested on yeast *Saccharomyces cerevisiae*. Results show that the pure *trans,trans* isomer has a higher antimicrobial activity than the mixture of isomers.

Keywords: Geometrical isomers; *Saccharomyces cerevisiae*; Sorbic acid; Organic acids

1. Introduction

Sorbic acid and its salts are widespread preservatives of foods with sufficiently low pH values to prevent bacterial but not yeast and mould growth. At the acidic pH, where sorbic acid is effective, the lipophilic undissociated molecule is freely permeable across the cell membrane. Subsequently, upon encountering the higher pH inside the cell, the molecule dissociates resulting in the release of charged anions and protons, which cannot cross the plasma membrane [1]. How dissociated acid affects viability of microorganisms is not understood in details. The inhibition of growth has been proposed to be due to a number of actions as membrane disruption [2], inhibition of essential metabolic reactions as glycolysis [3], induction of energetically expensive stress response [4,5] or accumulation of toxic anions [6].

Sorbic acid (2,4-hexadienoic acid) has two double bonds and can therefore adopt four geometrical isomers. The naturally occurring *trans,trans* isomer found in rowanberries [7] is used as a preservative. Other isomers — with the exception of *trans*-2,*cis*-4 hexadienoic acid — were not detected yet [8].

Our recent investigation of di-unsaturated decadienoic acid revealed that irradiation of the *trans,cis* isomer give rise to four different geometrical isomers [9,10]. As sorbic acid also posseses two double bonds, we expected that irradiation may accordingly

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lead to isomerisation. Therefore we have induced photochemical isomerisation of the *trans,trans* isomer of sorbic acid, its potassium salt and ethyl sorbate and analysed the reaction products.

Analysis of geometrical isomers is not an easy task due to the closely similar physical and chemical properties and therefore HPLC and GS–MS analyses were applied for separation and quantitative determination of isomers and NMR for their identification.

cis–trans Geometrical isomerisation of double bond may drastically change properties of the molecule. Pheromones as methyl 2,4-decadienoate, where the *trans,cis* isomer is significantly more active than any other isomer [11] and bombycol, where the naturally occurring *trans*-10,*cis*-12 isomer has an activity several orders of magnitude higher than that of other isomers [12], are known. However, there are many substances similar to sorbic acid, which have double bonds, but biological activity of isomers was not studied. The mixture with the defined composition containing all four isomers of potassium sorbate was tested as a preservative on *Saccharomyces cerevisiae* and its efficiency was compared to the *trans,trans* isomer of potassium sorbate.

2. Experimental

2.1. Chemicals

Sorbic acid (*trans*-2,*trans*-4-hexadienoic acid), its potassium salt (both 99+%) and ethyl sorbate (98%) were supplied by Sigma–Aldrich (Steinheim, Germany), ethanol (absolute, analytical grade) was from Merck (Darmstadt, Germany). The following solvents for HPLC analysis were used: ultra pure water (Milli-Q system, Millipore, Molsheim, France), trifluoroacetic acid (≥99%) from Fluka (Buchs, Switzerland) and methanol (HPLC grade) from Rathburn (Walkerburn, UK).

2.2. Solution preparation

Of the four possible isomers of the sorbates, only *trans*-2,*trans*-4-hexadienoates are commercially available. To produce the other isomers 150 ml of standard solutions (concentrations of potassium sorbate in water and sorbic acid in ethanol were 10 mg/ml, and concentration of ethyl sorbate in ethanol was 5 mg/ml) were exposed to UV irradiation from a 50 W Hg high-pressure lamp (Osram, Ultra-vitalux) for 120 min. The intensity of the incident light inside the photoreactor, measured using ferrioxalate actinometry, was $9.0 \cdot 10^{16}$ photons s^{-1}.

Solutions of 10 mg/ml of sorbic acid and its potassium salt in ethanol and water, respectively and 5 mg/ml of ethyl sorbate in ethanol before and after irradiation were used for NMR analysis. HPLC analyses were performed with the aqueous solutions of sorbates at a concentrations 100 μg/ml. The same concentration of ethyl sorbate in ethanol was used with GC–MS experiments.

2.3. High-performance liquid chromatography

The HPLC system (1100; Hewlett-Packard) consisted of a binary pump, a diode array detection (DAD) system and an injection valve (Model 8125; Rheodyne) with a 5-μl sample loop. The detector was set to a wavelength of 254 nm. Separations were made on a 3 μm ODS Hypersil column (100×2.1 mm I.D.; Hewlett-Packard) at a flow rate of 0.4 ml/min and at room temperature.

For isomers of sorbic acid and potassium sorbate the initial eluent comprised an aqueous solution of trifluoroacetic acid (concentration 1 g/l), and then changed linearly to a final composition of eluent: aqueous solution of trifluoroacetic acid–methanol (95:5, v/v), in 90 min. For the ethyl sorbate isomers the eluent contained methanol and purified water. The initial ratio of methanol–water (10:90, v/v) linearly changed to the final composition of methanol–water (30:70, v/v) in 60 min.

2.4. Gas chromatography

GC–MS analyses were performed on a Hewlett-Packard gas chromatograph HP 5890 Series II coupled to an HP 5989A mass spectrometer MS engine and equipped with a VOCOL, 60 m×0.25 mm I.D., 1.50 μm film thickness, capillary column (Supelco, Bellefonte, PA, USA). Helium (5.0, Messer Griesheim, Gumpoldskirchen, Austria) was used as the carrier gas at a flow rate of 1 ml/min. Injection (2 μl) was in the splitless mode, the purge was switched on after 1 min and the purge flow was

15 ml/min. The oven temperature program was: 80°C for 2 min, and then heated to 190°C at 3°C/min. The temperatures of the injector and GC–MS interface were 220 and 250°C, respectively. Ion source and quadrupole mass analyser temperatures were 200 and 100°C. The ionisation mode was electron impact with an electron energy of 70 eV.

2.5. NMR spectroscopy

NMR spectra were recorded on a Varian Unity Inova 600 spectrometer (^{1}H at 600.05 MHz) at the National NMR Centre of Slovenia. The samples of sorbic acid and ethyl sorbate were dissolved in ethanol with 10% of [$^2H_6$]ethanol added for lock. Potassium sorbate was dissolved in water with 10% of ^2H_2O added for a lock. The sample temperature was maintained at 25±0.5°C. The following parameters were used for data acquisition and processing of ^{1}H spectra: a WET solvent suppression technique was used for multiple suppression of the ethanol and water signals with selective SEDUCE pulse shape, 6.7 kHz sweep width, 90° pulse width, a pulse delay of 3 s, 64 scans, 32 kilobytes time domain, zero filling to 64 kilobytes and line broadening of 0.2 Hz prior to Fourier transformation. The assignments of the coupled and partly overlapped ethylene resonances in ^{1}H NMR spectra were made using detailed selective homonuclear decoupling experiments.

2.6. Microbiological conditions

The inhibitory activity of potassium sorbate isomers on microbial growth was tested with the type strain *Saccharomyces cerevisiae* ZIM 0753 (NRRL Y-12632). Growth experiments were performed aerobically on a rotary shaker (200 rpm) for 144 h at 28°C. Standard *trans,trans* potassium sorbate or a UV-treated mixture containing all four isomers of the preservative were added as a water solution to the final concentration of 1 mg/ml in the yeast growth medium just before the inoculation of yeast. The initial concentration of yeast was 10^5/ml. Samples of broth were taken just after inoculation and after 5, 24, 48, 72 and 144 h of growth, properly diluted, plated on OGY agar and counted as CFU/ml after 48 h of incubation at 28°C. Four to six plates with at least two different dilutions were counted. All results were calculated as the average values from at least two independent growth experiments.

3. Results and discussion

3.1. Isomerisation of trans,trans sorbates

Irradiation of standard solutions of *trans,trans* isomers of sorbic acid, potassium sorbate and ethyl sorbate (Fig. 1) resulted in isomerisation to all four geometrical isomers, accompanied by partial degradation. Irradiation time of 120 min was chosen due to the highest yield of formed isomers and large difference in their relative proportion, enabling one to distinguish between them. Shorter irradiation times led to a lower transformation of *trans,trans* isomers, while longer irradiation resulted in major degradation of all isomers.

Influence of temperature on potassium sorbate isomerisation was also examined. Heating of *trans,-trans* potassium sorbate granules at 200°C for 60 min resulted in a slight change of the colour, from white to brownish as reported previously [13]. Chromatographic analysis of heated sorbate revealed that only the *trans,trans* isomer was present in the unchanged amount, meaning that heating under these conditions does not result in considerable degradation and isomerisation of *trans,trans* isomer.

3.2. Chromatographic separation of sorbate isomers

Sorbic acid and its potassium salt isomers were successfully separated with HPLC. Fig. 2a shows HPLC chromatograms of standard solution of *trans,-trans*-sorbic acid before and after irradiation for 120 min. Retention times of potassium sorbate isomers

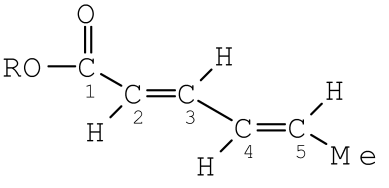

Fig. 1. Structural formula of *trans*, *trans* isomer of sorbic acid (R=H), its potassium salt (R=K) and ethyl ester (R=C_2H_5).

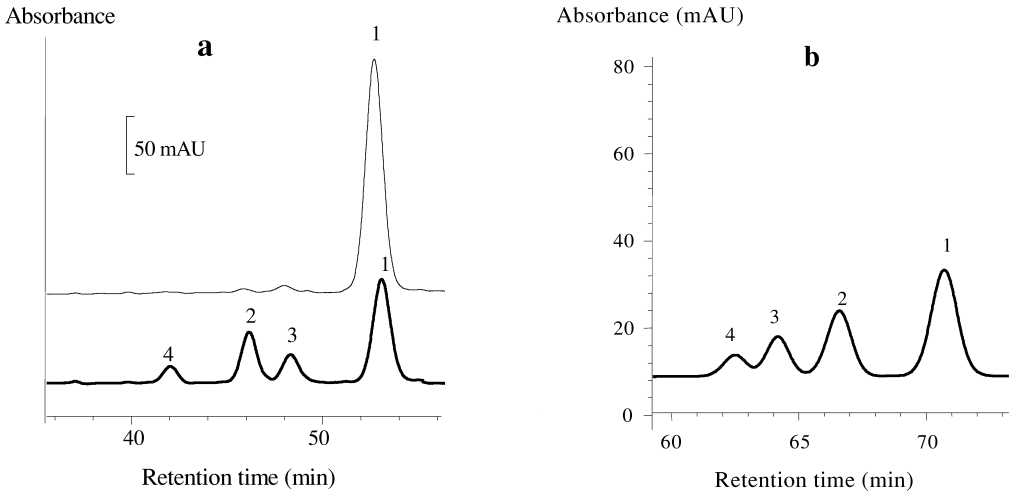

Fig. 2. HPLC chromatograms of sorbic acid (a) and ethyl sorbate isomers (b). Thin line represents solutions before irradiation and thick lines represents solutions after irradiation for 120 min. Peaks: 1=*trans*-2,*trans*-4 isomer, 2=*cis*-2,*trans*-4 isomer, 3=*trans*-2,*cis*-4 isomer and 4=*cis*-2,*cis*-4 isomer.

are identical to the isomers of sorbic acid (Fig. 2a), since separation was performed at acidic pH (trifluoroacetic acid).

Gas chromatographic analysis of potassium sorbate could not be performed due to the unvolatility of the salt and separation of sorbic acid isomers using gas chromatography was insufficient, as only two chromatographic peaks were observed on the VOCOL column (not shown). The ethyl ester of sorbic acid is more volatile and less polar as acid. Ethyl sorbate isomers, obtained after UV irradiation of *trans,trans*-ethyl sorbate, were therefore successfully separated with gas chromatography (Fig. 3). The ethyl sorbate isomers were also separated with HPLC (Fig. 2b), however with different solvents and gradients, compared to sorbic acid, due to lower polarity. The main advantage of GC–MS over HPLC analysis is, that the molecular peak $M^{+\cdot}$ and the fragmentation pattern are characteristic enough to distinguish ethyl sorbate isomers from other compounds, while UV spectra are not so characteristic. An advantage of GC–MS is also the shorter time of analysis (see Figs. 2b and 3).

However, mass spectra of geometrical isomers of ethyl sorbate were too similar to distinguish between them and no major differences could be observed in the UV spectra of sorbic acid, salt and ester isomers. Only the *trans,trans* isomer in all three forms (acid, salt and ester) was commercially available and its retention times could therefore be determined. In order to determine geometrical configuration of the other three isomers and their relative proportions after isomerisation the NMR spectra of potassium sorbate, sorbic acid and ethyl sorbate solutions were

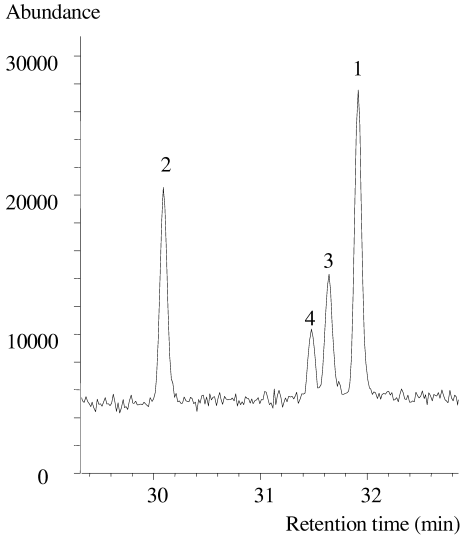

Fig. 3. GC–MS total ion current (TIC) chromatogram in the region of ethyl sorbate isomers. Peaks: 1=*trans*-2,*trans*-4 isomer, 2=*cis*-2,*trans*-4 isomer, 3=*trans*-2,*cis*-4 isomer and 4=*cis*-2,*cis*-4-isomer.

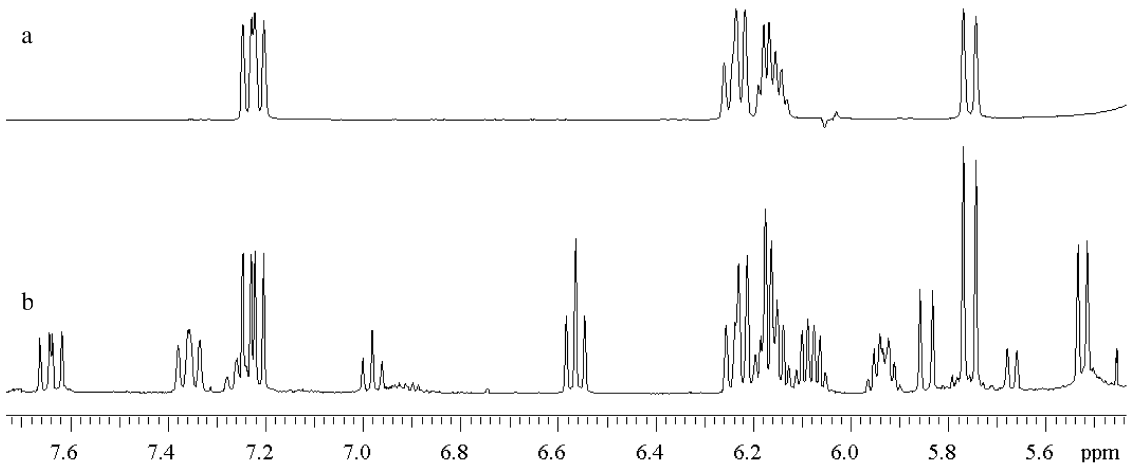

Fig. 4. NMR spectra of sorbic acid solution before (a) and after irradiation for 120 min (b). Assignments of proton signals are given in Table 1.

recorded before and after irradiation. Resolved chemical shifts of protons adjacent to the double bonds enabled qualitative and quantitative determination of isomers in the mixture (Fig. 4, Table 1). Comparison of relative areas of individual isomers obtained by GC and HPLC to the integrals in the ^{1}H NMR spectra (cf. Table 2) enabled structural characterisation of chromatographic peaks for all chromatographic analyses.

The variation in the relative areas of the same irradiated solutions determined by different methods (Table 2) is the largest for the *cis,cis* isomer which was present in the lowest concentrations and therefore subjected to the largest error. Slightly different molar extinction coefficients of isomers, variations in ionisation in the MS analysis and partial overlapping of resonances in NMR spectra can also give rise to differences in the estimation of relative contents [9].

GC–MS and HPLC can be used for quantitative analysis of sorbic acid and its derivatives. Linear range and limit of detection (LOD) (defined as triple standard deviation of noise peak areas expressed in

Table 1
^{1}H-NMR data for solutions of sorbic acid, its potassium salt and ethyl ester[a]

Compound	Geometrical configuration	Chemical shift (ppm)				Coupling constant (Hz)		
		δ_{H2}	δ_{H3}	δ_{H4}	δ_{H5}	J_{H2H3}	J_{H3H4}	J_{H4H5}
Sorbic acid	*trans*-2,*trans*-4	5.76	7.23	6.23	6.16	15.2	11.0	15.0
	cis-2,*trans*-4	5.53	6.57	7.36	6.09	11.5	11.4	14.8
	trans-2,*cis*-4	5.85	7.64	6.20	6.18	15.0	11.6	11.7
	cis-2,*cis*-4	5.67	6.99	7.28	5.94	12.0	11.6	9.1
Potassium sorbate	*trans*-2,*trans*-4	5.69	6.86	6.10	5.98	15.3	10.7	15.3
	cis-2,*trans*-4	5.49	6.21	6.83	5.87	11.4	11.5	15.7
	trans-2,*cis*-4	5.78	7.26	6.08	6.03	15.5	12.0	11.4
	cis-2,*cis*-4	5.68	6.57	6.69	5.74	11.3	11.8	10.5
Ethyl sorbate	*trans*-2,*trans*-4	5.78	7.25	6.28	6.21	15.7	10.5	15.0
	cis-2,*trans*-4	5.53	6.60	7.38	6.14	11.5	11.2	15.4
	trans-2,*cis*-4	5.88	7.66	6.19	6.24	15.7	11.9	10.3
	cis-2,*cis*-4	5.68	7.03	7.30	5.97	11.8	11.3	11.0

[a] Note: Shapes of the signals were: d for H2, dd for H3 and m for H4 and H5. Protons are marked according to the numbering of carbon atoms as shown in Fig. 1.

Table 2
Comparison of sorbic acid, its potassium salt and ethyl sorbate isomers content from NMR, HPLC and GC–MS analyses[a]

Compound	Geometrical configuration	Method		
		NMR	HPLC	GC–MS
Sorbic acid	*trans*-2,*trans*-4	1	1	–
	cis-2,*trans*-4	0.30	0.30	–
	trans-2,*cis*-4	0.22	0.19	–
	cis-2,*cis*-4	0.15	0.08	–
Potassium sorbate	*trans*-2,*trans*-4	1	1	–
	cis-2,*trans*-4	0.45	0.38	–
	trans-2,*cis*-4	0.27	0.21	–
	cis-2,*cis*-4	0.18	0.11	–
Ethyl sorbate	*trans*-2,*trans*-4	1	1	1
	cis-2,*trans*-4	0.60	0.64	0.60
	trans-2,*cis*-4	0.38	0.39	0.38
	cis-2,*cis*-4	0.28	0.20	0.28

[a] Note: Amounts of individual isomers are normalized with respect to the peak area of *trans,trans* isomer.

concentration units) of the chromatographic methods used were determined by analysing standard solutions of the commercially available *trans,trans* isomer of sorbic acid, its potassium salt and ethyl ester. The GC–MS response for ethyl sorbate is linear in the concentration range from 10 to 500 μg/ml and the LOD was found to be 2 μg/ml, while the HPLC response is linear from 5 to 150 μg/ml and the LOD is 0.5 μg/ml. The HPLC response for sorbic acid and potassium sorbate is linear from 10 to 200 μg/ml and the LOD is 1 μg/ml.

3.3. NMR characterisation of sorbate isomers

In the ^{1}H-NMR spectra of sorbic acid and potassium sorbate all ethylene proton signals are well resolved, whereas in the spectrum of ethyl sorbate the signals of H4 and H5 are partially overlapped. In the ^{1}H-NMR spectra of pure, non-irradiated *trans,-trans* isomers of potassium sorbate, sorbic acid and ethyl sorbate four characteristic ethylene signals were observed. In all three compounds the signal for H3 (doublet of doublet) is the most deshielded of all ethylene protons (Table 1). H2 (doublet) is upfield from H3 by $\Delta\delta$ of 1.1 to 1.5 ppm and is most upfield of all ethylene protons. The multiplets of H4 and H5 are found between 5.9 and 6.2 ppm (Table 1). Methyl groups are doublets with chemical shifts of 1.85 ppm. The individual $^3J_{HH}$ proton–proton coupling constants were extracted and are given in Table 1. The key J_{H2H3} and J_{H4H5} coupling constants were found in the range from 15.0 to 15.7 Hz, which proved *trans* configurations along the C2=C3 and C4=C5 double bonds (Table 1, Fig. 4a).

^{1}H-NMR spectra of irradiated solutions of potassium sorbate, sorbic acid and ethyl sorbate revealed several additional signals compared to ^{1}H-NMR spectra of pure *trans,trans* isomers due to isomerisation along the C2=C3 and C4=C5 double bonds (Fig. 4b). The resonances corresponding to all three additional geometrical isomers, *trans–cis*, *cis–trans* and *cis–cis* could be identified together with the parent *trans–trans* isomer (Table 1). The four isomers were identified on the basis of their characteristic key $^3J_{HH}$ coupling constants along the C2=C3 and C4=C5 double bonds. For the *cis* configuration J_{H2H3} and J_{H4H5} values are between 9.1 and 12.0 Hz, whereas they are between 14.8 and 15.7 Hz for *trans* configuration, which is in agreement with the literature data [14]. The signals for H3 in C2-*trans* isomers are deshielded from the corresponding C2-*cis* isomers of potassium sorbate, sorbic acid and ethyl sorbate by $\Delta\delta$ of ca. 0.6 ppm in both C4-*cis* and C4-*trans* isomers (Table 1). Similar downfield shift of H2 resonances in C2-*trans* isomers with respect to their C2-*cis* counterparts is uniformly observed, but is smaller than for H3 protons with $\Delta\delta$ between 0.2 and 0.3 ppm. The signals for H4 in C4-*trans* isomers are uniformly deshielded from the corresponding C4-*cis* isomers by $\Delta\delta$ of $<$0.1 ppm in both C2-*cis* and C2-*trans* isomers of potassium sorbate, sorbic acid and ethyl sorbate (Table 1). It is noteworthy that isomerisation across the C2=C3 double bond has a large effect on chemical shielding of H4. In all C2-*cis* isomers H4 is deshielded by $\Delta\delta$ from 0.7 to 0.9 ppm in comparison to C2-*trans* isomers which can be nicely attributed to the fact that H4 is in C2-*cis* isomers in close proximity to the carbonyl group and thus experiences the anisotropy of its chemical shielding zone. In C2-*trans* isomers of potassium sorbate, sorbic acid and ethyl sorbate H5 is found to be slightly deshielded by $\Delta\delta$ of $<$0.05 ppm in C4-*cis* isomers in comparison to C4-*trans* isomers. In C2-*cis* isomers, however, H5 protons in C4-*trans* isomers exhibit downfield $\Delta\delta$

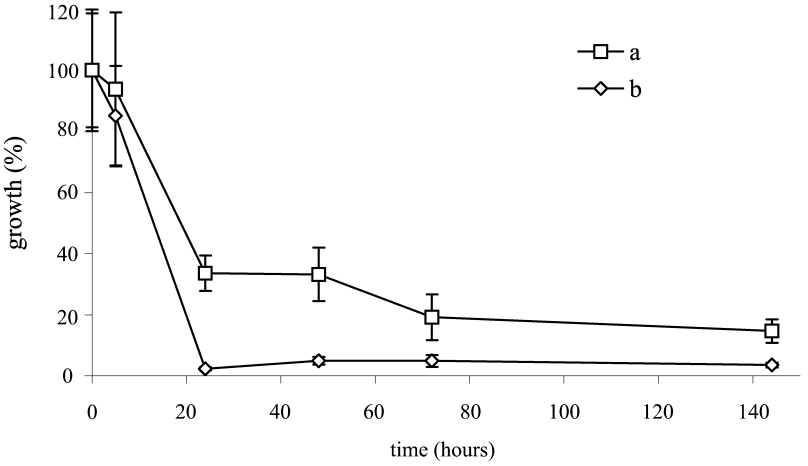

Fig. 5. Relative growth of *Saccharomyces cerevisiae* in the presence of pure (non-irradiated) *trans,trans*-potassium sorbate (b) and in the presence of a mixture of potassium sorbate isomers (120 min irradiated solution of pure *trans,trans*-potassium sorbate) (a) compared to normal growth of yeasts without addition of preservative.

values between 0.13 and 0.17 ppm in comparison to C4-*cis* isomers (Table 1).

3.4. Microbiological assay

Analysis of photochemically induced isomerisation of potassium *trans,trans* sorbate after 120 min of irradiation revealed that ~40% is transferred to the other three isomers (Table 2). In order to check whether antimicrobial activity is affected by isomeric composition, yeast growth in the presence of pure *trans,trans*-potassium sorbate and irradiated potassium sorbate were compared.

Irradiation results not only in isomerisation, but also in partial degradation. Therefore, it was necessary to concentrate the irradiated solution on rotavapor and re-adjust the concentration of all the isomers to the concentration of the *trans,trans* isomer in non-irradiated solutions. Total amount and proportion of isomers remained constant during microbiological experiments as determined by HPLC.

The comparison of growth curves indicates that *trans,trans* isomer is much more effective in inhibiting yeast growth than the mixture of all four geometrical isomers (Fig. 5). Apparently lower antimicrobial activity of isomers with at least one *cis* double bond could be correlated to the lower permeability of *cis* isomers across the membrane, due to steric constraints [15], resulting in relatively lower intracellular concentration compared to the *trans,-trans* isomer. A different effect on the putative intracellular target due to the isomerism could also not be excluded.

Sorbate added to food and beverages, especially those stored in transparent flasks, could isomerase, if exposed to the sunlight radiation for a prolonged time. These products should be critically examined concerning isomerisation especially when sorbate is added in minimal quantities.

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