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Study of antioxidant and mutagenic activity of different orange juices

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Abstract

The mutagenic effects of *in natura* and processed (fresh and frozen) orange juices were evaluated by the *Salmonella* /microsome assay (Ames Test). Antioxidant potential was determined by deoxyribose degradation, as well as by the nitro blue tetrazolium reduction test. The juices inhibited degradation of deoxyribose and were able to trap the superoxide anion. At concentrations up to 1% they acted as pro-oxidants in lipid peroxidation in a concentration-dependent manner. However, this activity was less effective at 10% juice concentration. The sweetened processed juice was not able to decrease peroxidation nor to trap superoxide anions. Significant correlation between total phenolics and the inhibition of deoxyribose degradation was observed; also, a significant correlation between lipid peroxidation and total phenolics was found. Vitamin C was a pro-oxidant in tests employing transition metals. Mutagenicity was observed in the Ames test, particularly for fresh, *in natura*, juice samples. The highest responses were observed in strains TA97a and TA98. Fresh processed juice, which had the best antioxidant potential, was not mutagenic in any of the strains tested. Positive results for mutagenesis in TA97a, with metabolization, were correlated with total phenolics and vitamin C.

Keywords: Orange juice; Mutagenicity; Ames test; Antioxidant and pro-oxidant activity; Vitamin C; Phenolic compounds

1. Introduction

Most free radical reactions involve the reduction of molecular oxygen, leading to the formation of reactive oxygen species (ROS), including superoxide anion and hydroxyl radical. These oxygen species can cause oxidative damage to several cell components and may thus play an important role in various pathological conditions. The inflicted damage may contribute to aging and to degenerative diseases such as brain dysfunction, cataracts, cancers, and cardiovascular diseases (Ko, Cheng, Lin, & Teng, 1998). Therefore, natural antioxidants present in orange juice can neutralize free radicals, due to their ability to act as free radical scavengers and/or as metal chelators (Aruoma, 1999; Wang, Cao, & Prior, 1996). However, many antioxidants have also been identified as natural mutagens and carcinogens, apart from their role as natural antimutagens and anticarcinogens (Ames, 1983; Middleton, Kandaswami, & Theoharides, 2000; Stavric, 1994).

Vitamin C and phenolic compounds are important antioxidants present in orange juice (Wang et al., 1996). They may act as antioxidants and as radical scavengers. The metal-binding ability of phenolic compounds has been shown to inhibit formation of hydroxyl radicals in the Fenton reaction by complexing ferrous ions. However, vitamin C and phenolic compounds, in the presence of Cu (II) and Fe (III), can also cause DNA degradation through generation of ROS, i.e. hydroxyl radicals (Ferguson, 2001; Stadler, Turesky, Müller, Markovic, & Leong-Morgenthaler, 1994; Wang et al., 1996). These radicals can either interact directly with

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DNA or may facilitate redox cycling, interacting with molecular oxygen to produce oxidative stress-generating and genotoxic ROS, that may cause DNA damage (Patrineli, Clifford, & Ionnides, 1996b).

The aim of this study was to investigate the mutagenic/genotoxic effects of *in natura* and processed, fresh and frozen orange juices by using the *Salmonella*/microsome assay (Ames Test), as well as to investigate their antioxidant properties by in vitro biochemical analysis. Also, the presence of organophosphorus and carbamate pesticides in the juices was determined, as these substances have been associated with mutagenicity in the Ames test (Hübner et al., 1997; Sierra-Torres, Cajas-Salazar, Hoyos, & Zuela Whorton, 1998). Finally, we also measured the amounts of vitamin C and of total phenolic compounds in all orange juice samples.

2. Materials and methods

2.1. Oranges and processed orange juices

The *in natura* oranges, cultivated without synthetic pesticides (organic), were obtained from the Cooperativa Ecológica Coolméia Ltda of Porto Alegre, RS, Brazil. Processed orange juices were bought at supermarkets, observing the expiry dates and keeping the same trademarks throughout the tests.

2.2. Sample preparation

Organic fresh and Organic frozen juices were prepared by washing the oranges, dipping them in 70% ethanol and flaming them at the Bunsen burner for about 5 s for sterilization. The oranges were then cut in half with a sterilized knife. Sterile crusher and sieve were employed for the preparation of the orange juice. An 1 ml aliquot was used immediately for the Ames test; the remaining juice was frozen at −20 °C and labeled "Org.frozen". The freshly processed orange juice samples were utilized immediately or frozen at −20 °C.

Fresh orange juices were of 4 types:

Org.fresh: organic *in natura* fresh orange juice.

Proc.fresh1: freshly processed orange juice that needs refrigeration, with a shelf life of 30 days (Tetra Pak).

Proc.fresh2: freshly processed orange juice that does not need refrigeration (stored at room temperature), with a usual shelf life of 365 days, sweetened (Tetra Pak).

Proc.fresh3: freshly processed orange juice that needs refrigeration, with a usual shelf life of 20 days (plastic package). Frozen orange juices were of the same classes and have their name changed in that fresh is substituted by frozen, e.g., *Org. frozen*, *Proc.frozen1* (Tetra Pak), *Proc.frozen2* (Tetra Pak), and *Proc.frozen3* (plastic package).

2.3. Microsome fraction

The post-microsomal fraction S9 was prepared from livers of Sprague–Dawley rats pre-treated with polychlorinated biphenyl mixture (Aroclor 1254), purchased from Molecular Toxicology Inc. (Moltox™). The S9 metabolic activation mixture was prepared according to Maron and Ames (1983) and Mortelmans and Zeiger (2000). All reagents were from Sigma.

2.4. Salmonella/microsome assay (Ames test)

Mutagenicity was determined in the pre-incubation procedure (Maron & Ames, 1983), using various concentrations of juices, with *Salmonella typhimurium* strains TA98, TA97a, TA100, and TA102, kindly provided by Dr. Bruce Ames (Dept. of Biochemistry, University of California, USA) (Levin, Hollstein, Christman, Schiwiers, & Ames, 1984), with or without S9 mix. The mixture, consisting of the juice samples to be tested, 500 μl of S9 mix (in test with metabolization) and 100 μl of the bacterial suspension ($1–2 \times 10^9$ cells/ml) was pre-incubated for 20 min at 37 °C without shaking. Then, 2000 μl of top agar (0.55% agar, 0.55% NaCl, 50 μM L-histidine, and 50 μM biotin, at pH 7.4, 45 °C) were added to the test tube and poured onto minimal agar (1.5% agar, Vogel-Bonner E medium, containing 2% glucose). All assays were carried out in triplicate.

After incubation for 48 h, colonies (His^+ revertants) were counted and the results were expressed as mutagenic index (MI = # of His^+ induced in the sample/# of spontaneous His^+ in the negative control).

Negative (appropriate solvent) and positive (10 μg sodium azide per plate for strain TA100; 5 μg 4-nitroquinoline 1-oxide per plate for strains TA98, TA97a, and TA102) controls were included in each assay. Aflatoxin B1 (10 μg per plate) was used as positive control for the metabolic activation for all strains.

A compound was considered positive for mutagenicity when (a) the number of revertants was at least double the spontaneous yield (MI $\geqslant$ 2), (b) a significant response for analysis of variance ($p \leqslant 0.05$) was found, and (c) a corresponding positive dose–response curve ($p \leqslant 0.01$) was present, as evaluated by the Salmonel software (Myers, Adams, Kier, Rao, Shaw, & Williams, 1991).

2.5. Determination of total phenolics, vitamin C, and pesticides

The total amount of phenolic compounds in the orange juices was determined according to the Folin-Ciocalteu procedure (Singleton & Rossi, 1965). Atleast 2 orange juice samples (100 μl each) were introduced into test tubes; 250 μl of Folin-Ciocalteu's reagent and 500 μl

of sodium carbonate were added and the tubes vigorously shaken. After standing for 40 min, absorption at 750 nm was measured. The total phenolics content was derived by comparison with a gallic acid standard curve (Sigma Co., 0.2–1 mg/ml gallic acid) and expressed as μg/ml gallic acid equivalents (GAE).

Vitamin C (ascorbic acid) was determined according to the Kabasakalis, Siopidou, and Moshatou (2000) at the University of Santa Cruz, RS (UNISC/Brazil). Organophosphorus and carbamate pesticides in the juice samples were determined as methyl parathion-equivalent activity, which causes inhibition of the enzyme acetylcholinesterase (AChE), as previously described by Bastos, Cunha, and Lima (1991) and Lima, Cunha Bastos, and Cunha Bastos (1996). A calibration curve of methyl parathion (Folidol 600® – Bayer) was used to express AChE activity in ppm of methyl parathion.

2.6. Deoxyribose assay

ROS used for the detection of oxidative damage to deoxyribose were generated according to Nishida, Yoshizawa, and Akamatsu (1991). Oxidative products, caused by the formation of thiobarbituric acid-reactive substances (TBARS), were assayed as described by Halliwell and Gutteridge (1981). Assays were in triplicate, in a final volume of 1.2 ml. 120 μl of the different concentrations of orange juices were added to every test tube before adding hydrogen peroxide. The absorbance of each assay was measured against a specific blank, with the same juice concentration as used in the assay. In this system, mannitol (1mM) was used as the positive control.

2.7. Superoxide anion scavenging activity

The phenazine-methosulfate(PM)-NADH method (Robak & Gryglewski, 1988) was used for the generation of $O_2^{\cdot -}$. The test tubes contained 12 μM PM, 100 μM NADH, and 100 μM NBT in 0.1 M phosphate buffer (K_2HPO_4–KH_2PO_4) at pH 7.8. After 2 min of incubation at room temperature, 100 μl of HCl 0.1 M were added to stop the reaction. The spectrophotometric measurement was at 560 nm against blank samples, in the absence of PM. Different concentrations of orange juices were added to the test tubes before adding PM. Superoxide dismutase (SOD) (100 U/ml) enzyme was used as a positive control.

2.8. Oxidation of methyl linoleate

Protecting activity against oxidative damage caused to methyl linoleate (MeLo) was measured by a modified method of Osawa, Katsuzaki, Hagiwara, Hagiwara, and Shibamoto (1992). Oxidative damage was induced by $CuSO_4$ (5 mM). The tubes contained phosphate buffer (0.01 M, pH 7.4), MeLo (14 mM), 10% SDS and $CuSO_4$ (5 mM). 120 μl of the different concentrations of orange juices were added to the test tube containing the previously added reaction mixture. The tubes were incubated for 20 h at 37 °C, with agitation. Then, 1 ml of 1% thiobarbituric acid (TBA) and 1 ml of 2.8% trichloroacetic acid (TCA) were added and this mixture was further incubated for 15 min at 100 °C for development of pigmentation. The reaction was stopped by chilling in ice for 5 min. After centrifugation, the absorbance of the clear supernatant was measured at 532 nm, against blank samples not containing MeLo and SDS. The absorbance of each assay was measured against a specific blank, containing with same juice concentration as used in the assay. Betahydroxytoluene (10 mM) was used as a positive control.

2.9. Statistical analysis

Spearman's (r_s) correlation coefficient was calculated with the SPSS/PC© programme/version 98. Differences at the $p \leqslant 0.05$ level were considered significant. Results obtained for the antioxidant effect of juice were analyzed by the ANOVA (analysis of variance) statistics programme. Difference between each juice and its control (100% oxidation) was evaluated by the Tukey test and differences at the $p \leqslant 0.01$ level were considered significant. The results for the antioxidant effect and for oxidation of MeLo, caused by different juice samples (fresh and frozen) at the same dilution, were evaluated by Student's t test and the level of significance considered was $p \leqslant 0.01$. Differences between fresh and frozen forms of the same juice were evaluated by t test and considered significant at the $p \leqslant 0.01$ level.

3. Results

The frozen and fresh forms of the same juice showed similar concentrations of total phenolic compounds except for *Proc.frozen1* and *Proc.fresh1* juices ($p \leqslant 0.01$, Table 1). The vitamin C concentrations of *Proc.frozen1* and *Proc.frozen3* juices were nearly 50% lower than that found in the respective processed fresh *Proc.fresh1* and *Proc.fresh3* juices. All other juices had no significant variation of vitamin C between fresh and frozen forms (Table 1). No preservatives, such as sorbic and benzoic acids, or sulfites were detected in the fresh juice samples (data not shown). Only *Proc.fresh1* had traces of methyl parathion-equivalent (<0.02 ppm, Table 1).

As shown in Fig. 1, all juices inhibited oxidative damage to deoxyribose in a dose-dependent manner. A similar protection was seen in the positive mannitol control (1 mM). At a juice concentration of 10%, the protective effect was in the order of 65–85%, and at 1% juice concentration it ranged from 60% to 83%. No

Table 1
Chemical analysis of *in natura*, processed, fresh and frozen orange juices

Juices	Vitamin C (mg/100 ml)	Total phenolics (μg/ml GAE[a])	Pesticides (ppm of methyl parathion-equivalents)
Org.frozen	65.2	598 ± 31.4	ND
Org.fresh	56.4	580 ± 132	ND
Proc.frozen1	16.7	$557 \pm 4.73^{*}$	ND
Proc.fresh1	45.8	$439 \pm 4.35^{*}$	<0.02
Proc.frozen2	42.3	550 ± 7.00	ND
Proc.fresh2	47.6	548 ± 3.96	ND
Proc.frozen3	26.4	534 ± 61.61	ND
Proc.fresh3	53.7	581 ± 10.8	ND

[a] Corresponds to the phenolic concentration expressed in μg/ml equivalent to gallic acid.
[*] $p < 0.01$ (test *t*); ND, not detected.

significant differences were observed between frozen and fresh juices, except between *Proc.fresh1* and *Proc.frozen1*. The *Proc. fresh1* juice gave the highest protection against oxidative damages to deoxyribose when compared to its frozen *Proc.frozen1* form at concentrations of 1% and 0.1% (Fig. 1(b)).

For the NBT reduction assay, which evaluates the $O_2^{\cdot -}$ scavenging ability, our results show that, with the exception of *Proc.fresh2* (Fig. 2(c)), all tested juices inhibited NBT reduction of 42–70% at the highest concentration tested (10%) (Figs. 2(a), (b), and (d)). Fig. 2 shows significant differences in the $O_2^{\cdot -}$ trapping ability between fresh and frozen samples (Figs. 2(b) and (d)) at the 10% level.

Up to a concentration of 1%, all juices caused oxidative damage to MeLo, showing a significant increase in lipid peroxidation (Fig. 3). However, at 10% concentration, *Org.fresh* and *Org.frozen in natura* juices, as well as the processed ones in the fresh forms (*Proc.fresh1* and *Proc.fresh3*), caused lower lipid peroxidation than the 1% samples (Figs. 3(a), (b) and (d)), though this reduction of MeLo peroxidation did not reach the level of the positive control [(−76%) 10 mM BHT]. Clearly, lipid peroxide increased significantly at all juice concentrations. The extent of peroxidation varied with fresh juice more effective in Fig. 3(b) and frozen was more effective in Figs. 3(a) and (d).

Spearman's (r_s) correlation coefficient showed that the total phenolic compounds of the juice samples were correlated to the lipid peroxidation level ($r_s = 0.857$). However, no significant correlation was detected in relation to vitamin C ($r_s = 0.690$). The concentration of phenolic compounds showed correlation with the antioxidant activity in the deoxyribose degradation ($r_s = -0.857$) and no correlation with NBT-reduction ($r_s = 0.143$) assays (data not shown).

As shown in Table 2, *Org.fresh*, *Org.frozen*., and *Proc.frozen1* juices, both in the presence and absence of metabolic activation, had mutagenic activity in strain

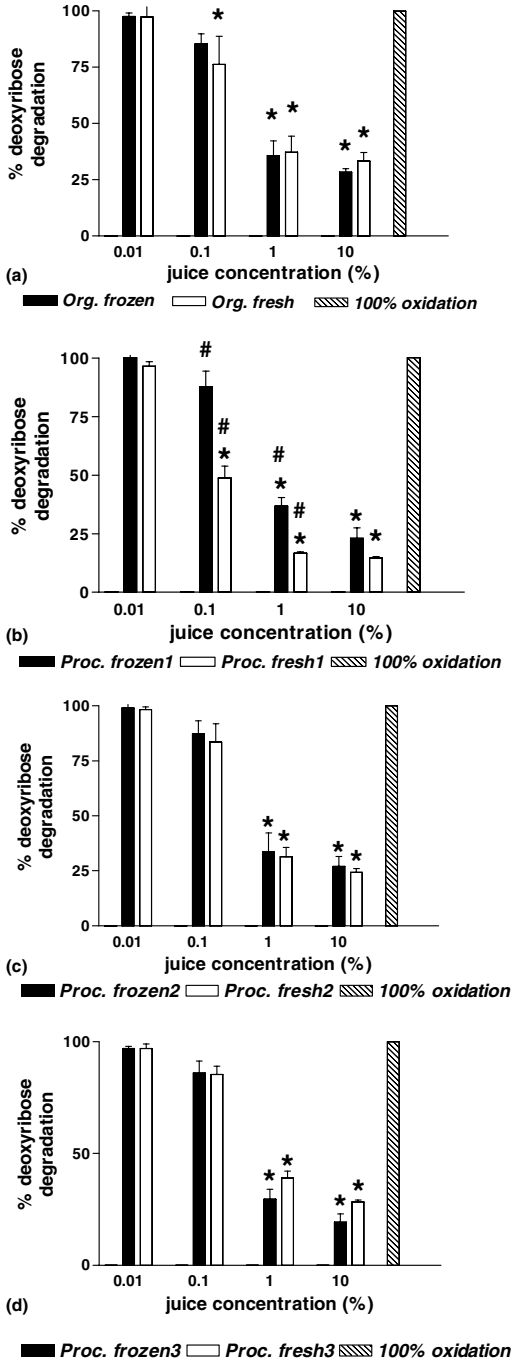

Fig. 1. Effect of in natura (a) and processed (b–d) orange juices on the degradation deoxyribose (Fe^{3+}–NTA–H_2O_2 system). Mannitol (1 mM), positive control, inhibited around 71% of deoxyribose degradation. $*p < 0.001$ (ANOVA/Tukey) – difference of each juice in relation to its control (100% oxidation); $\#p < 0.01$ (Test *t*, Student) – difference between fresh and frozen juices at the same concentration.

TA98, which detects frameshift mutations (Maron & Ames, 1983). The *Proc.frozen2* and *Proc.fresh2* juices were mutagenic in the absence of metabolic activation in strain TA98. However, *Proc.frozen3* juice required metabolization in order to cause frameshift mutation in

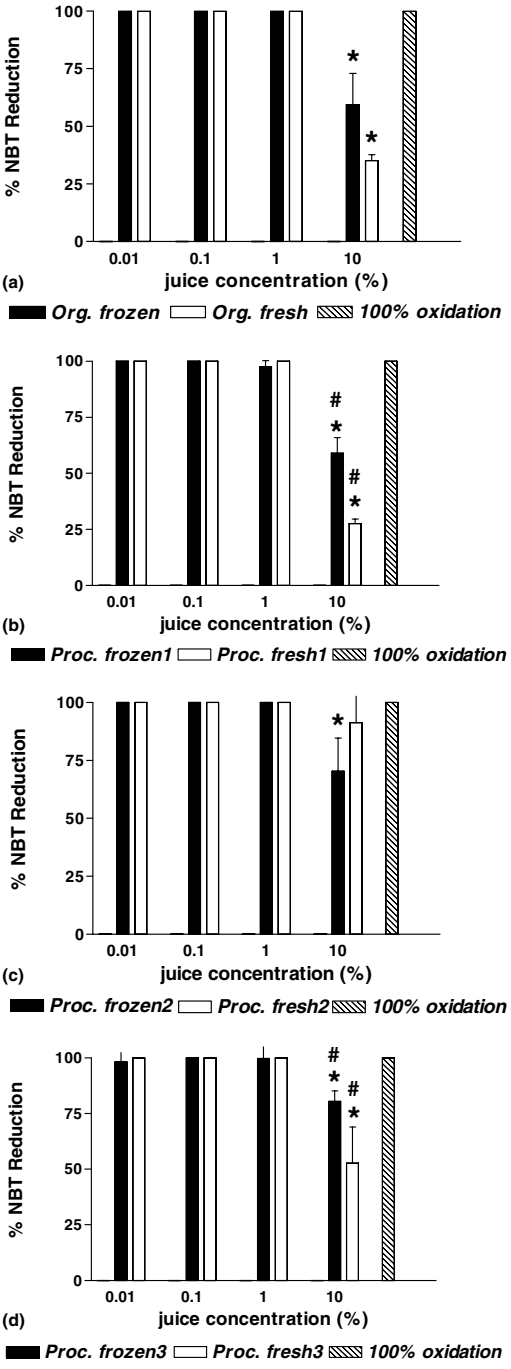

Fig. 2. Effect of *in natura* (a) and processed (b–d) orange juices on the NBT reduction (phenazine–NADH methosulfate system). SOD (100 U/ml) positive control, inhibited around 75% the NBT-reduction. $*p < 0.001$ (ANOVA/Tukey) – difference of each juice in relation to its control (100% oxidation); $\#p < 0.01$ (Test *t*, Student) – difference between fresh and frozen juices in relation to the same concentration.

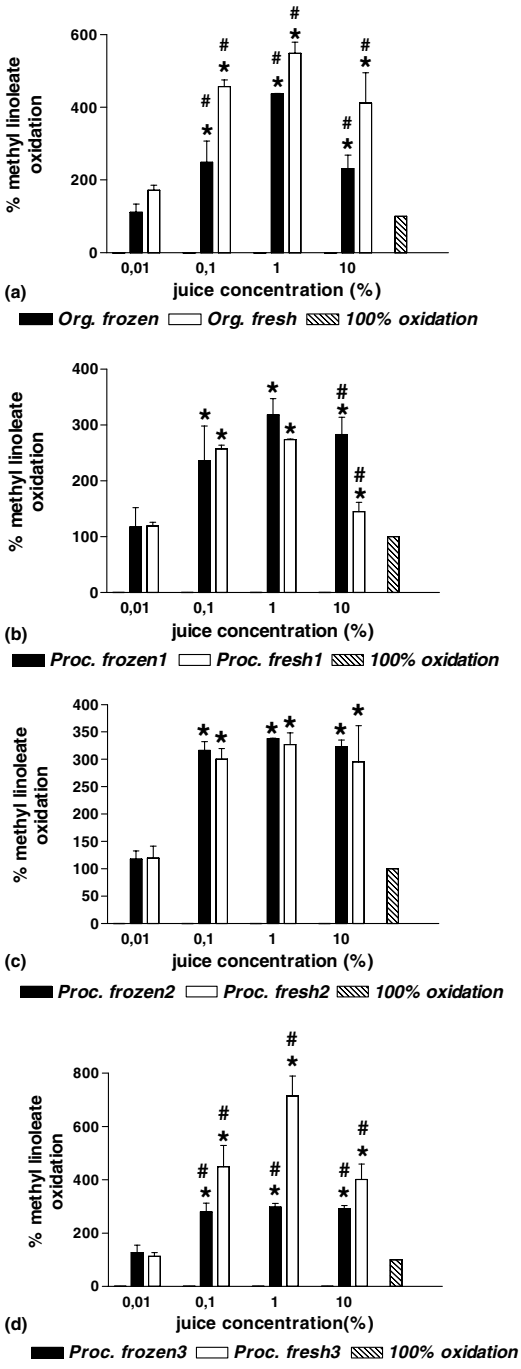

Fig. 3. Effect of *in natura* (a) and processed (b–d) orange juices on $CuSO_4$-induced lipid peroxidation. BHT (10 mM), positive control, inhibited around 76% the peroxidation. $*p < 0.001$ (ANOVA/Tukey) – difference of each juice in relation to its control (100% oxidation); $\#p < 0.01$ (Test *t*, Student) – difference between fresh and frozen juices in relation to the same concentration.

TA98 (Tables 2 and 4). The *Org.fresh* juice was mutagenic in strain TA97a, both in the presence and absence of metabolic activation (Tables 2 and 4). The *Proc.fresh3* and *Proc.frozen1* juices were able to induce frameshift mutation only in the absence of metabolic activation (Tables 2 and 4). In strain TA100, only the *Org.fresh* juice, both in the presence and absence of metabolization, was able to induce base-pair substitutions (Tables 3 and 4). When orange juices were tested in TA102, which detects oxidative and alkylating mutagens

Table 2
Induction of His^+ revertants in *S. typhimurium* TA97a and TA98 by fresh and frozen orange juices, without (−S9) and with (+S9) metabolic activation

Spontaneous revertants		*Salmonella typhimurium* strains							
		TA98				TA97a			
		−S9		+S9		−S9		+S9	
		15 ± 6		26 ± 14		104 ± 16		115 ± 18	
Juices	Conc. (μl)	(a)	IM[b]	(a)	IM[b]	(a)	IM[b]	(a)	IM[b]
Org.fresh	0*	15 ± 5		12 ± 4		61 ± 27		75 ± 4	
	100	24 ± 4	1.57	25 ± 7	2.03	65 ± 10	1.05	91 ± 7	1.21
	500	24 ± 12	1.54	28 ± 8	**2.30**	86 ± 6	1.40	104 ± 3	1.38
	2000	52 ± 9	**3.41**	52 ± 6	**4.19**	144 ± 21	**2.34**	151 ± 13	**2.00**
Org.frozen	0	14 ± 6		20 ± 4		77 ± 12		94 ± 14	
	100	18 ± 1	1.31	23 ± 6	1.15	68 ± 24	0.99	101 ± 11	1.08
	500	18 ± 6	1.31	24 ± 3	1.20	60 ± 27	0.79	103 ± 3	1.10
	2000	78 ± 20	**5.57**	87 ± 27	**4.37**	99 ± 21	1.30	143 ± 22	1.52
Proc.fresh1	0	22 ± 2		26 ± 5		61 ± 27		68 ± 29	
	100	16 ± 9	0.70	24 ± 12	0.91			76 ± 10	1.11
	500	15 ± 5	0.67	35 ± 16	1.33			72 ± 9	1.06
	2000	13 ± 3	0.58	14 ± 7	0.53			50 ± 25	0.70
Proc.frozen1	0	11 ± 3		19 ± 5		122 ± 4		115 ± 12	
	50							95 ± 5	0.82
	75							94 ± 13	0.81
	100	10 ± 1	0.96	19 ± 3	1.02	106 ± 9	0.87	81 ± 2	0.70
	500	49 ± 2	**4.57**	38 ± 17	1.50	137 ± 7	1.12	104 ± 11	0.90
	2000	54 ± 13	**5.00**	35 ± 18	**2.44**	265 ± 35	**2.18**		
Proc.fresh2	0	4 ± 1		23 ± 7		113 ± 4		105 ± 13	
	100	6 ± 3	1.38	28 ± 2	1.23	107 ± 28	0.95	107 ± 20	1.02
	200			39 ± 7	1.71				
	300			23 ± 9	1.01				
	400			34 ± 6	1.55				
	500	10 ± 5	**2.23**			91 ± 3	0.81	106 ± 8	1.01
	1000								
	1500								
	2000	21 ± 3	**4.85**			62 ± 15	0.55	122 ± 3	1.16
Proc.frozen2	0	10 ± 1		51 ± 7		143 ± 13		214 ± 8	
	100	12 ± 6	0.83	20 ± 3	1.13	140 ± 24	0.98	176 ± 35	0.82
	500	14 ± 1	1.40	13 ± 9	0.77	189 ± 27	1.32	178 ± 23	0.83
	1000					194 ± 12	1.36		
	1500								
	2000	27 ± 4	**2.70**	12 ± 4	0.70	214 ± 4	1.50	153 ± 9	0.72
Proc.fresh3	0	15 ± 4		16 ± 4		8 ± 6		61 ± 16	
	25					73 ± 7	9.16		
	50					19 ± 27	3.16		
	75					42 ± 9	**5.20**		
	100	9 ± 4	0.56	23 ± 5	1.42	41 ± 4	**5.12**	52 ± 17	0.86
	500	17 ± 5	1.09	20 ± 4	1.23			94 ± 6	1.55
	2000	17 ± 8	1.09	22 ± 15	1.38			77 ± 21	1.27
Proc.frozen3	0	13 ± 4		13 ± 2		94 ± 11		238 ± 6	
	100	11 ± 2	0.84	18 ± 6	1.37	64 ± 11	0.68	238 ± 54	1.00
	500	6 ± 1	0.50	26 ± 1	**1.98**	65 ± 10	0.70	218 ± 12	0.92
	2000	13 ± 2	1.00	32 ± 1	**2.40**	114 ± 20	1.22	132 ± 19	0.55
4NQO	5 μg	246 ± 135				647 ± 325			
AFB_1	10 μg			323 ± 200				562 ± 186	

(a): Number of revertants. His+/plate.
[b] Mutagenic index: no. of His^+ induced in the sample/no. of spontaneous His^+ in the negative control.
* O: Negative control: distilled water; positive control: (−S9) 4-nitroquinoline 1-oxide (4NQO; 5 μg/plate) for both strains; (+S9) aflatoxin B_1 (10 μg/plate) for all strains. Results in bold: positive mutagenics (ANOVA, $p \leqslant 0.05$; dose–response curve, $p \leqslant 0.01$; MI $\geqslant 2$).

and active forms of oxygen (Levin et al., 1984), no positive response was observed, regardless of microsome activation (Tables 3 and 4).

The observed mutagenicity might be due to histidine residues in some of the juice samples which would, by allowing growth of the auxotrophic test bacteria on the

Table 3
Induction of His^+ revertants in *S. typhimurium* TA100 and TA102 by fresh and frozen orange juices, without (−S9) and with (+S9) metabolic activation

Spontaneous revertants		*Salmonella typhimurium* strains							
		TA100				TA102			
		−S9		+S9		−S9		+S9	
		133 ± 27		121 ± 26		241 ± 51		282 ± 52	
Juices	Conc. (μl)	(a)	IM[b]	(a)	IM[b]	(a)	IM[b]	(a)	IM[b]
Org.fresh	0*	138 ± 20		118 ± 13		224 ± 42		271 ± 13	
	100	155 ± 13	1.12	127 ± 5	1.07	181 ± 21	0.81	257 ± 8	0.95
	500	177 ± 1	1.28	167 ± 22	1.41	193 ± 6	0.86	253 ± 41	0.94
	2000	312 ± 28	**2.26**	280 ± 7	**2.37**	239 ± 54	1.07	383 ± 81	1.41
Org.frozen	0	77 ± 13		94 ± 14		14 ± 6		20 ± 4	
	100	102 ± 22	1.04	159 ± 68	1.03	266 ± 74	0.92	258 ± 24	0.89
	500	105 ± 13	1.07	118 ± 8	0.77	202 ± 14	0.70	227 ± 37	0.79
	2000	98 ± 19	1.00	255 ± 62	1.65	307 ± 28	1.06	273 ± 21	0.94
Proc.fresh1	0	145 ± 24		119 ± 14		258 ± 14		195 ± 23	
	100	115 ± 45	0.80	109 ± 8	0.92	172 ± 28	0.66	202 ± 7	1.04
	500	99 ± 8	0.68	153 ± 27	1.29	174 ± 38	0.67	227 ± 30	1.16
	2000	131 ± 61	0.90	131 ± 30	1.11	47 ± 30	0.18	216 ± 0	0.94
Proc.frozen1	0	165 ± 38		159 ± 32		271 ± 8		271 ± 20	
	50	131 ± 24	0.79						
	75	135 ± 17	0.81						
	100	139 ± 15	0.84	213 ± 14	1.34	281 ± 16	1.04	253 ± 45	0.94
	500	137 ± 16	0.83	223 ± 40	1.41	373 ± 19	1.38	251 ± 27	0.93
	2000			182 ± 57	1.15	381 ± 6	1.41	256 ± 50	0.95
Proc.fresh2	0	162 ± 8		105 ± 29		167 ± 8		238 ± 29	
	100			251 ± 50	1.05	162 ± 2	0.97	251 ± 50	1.05
	500					130 ± 4	0.78	279 ± 44	1.17
	1000	119 ± 18	0.74	88 ± 25	0.84				
	1500	145 ± 24	0.89	152 ± 29	1.45				
	2000	163 ± 26	1.00	164 ± 4	1.56	115 ± 23	0.69	155 ± 19	0.65
	2500	157 ± 3	0.96						
Proc.frozen2	0	152 ± 19		153 ± 18		270 ± 19		317 ± 21	
	50	150 ± 7	0.99						
	75	127 ± 10	0.84						
	100	119 ± 12	0.78			215 ± 4	0.79	311 ± 6	0.98
	500	116 ± 11	0.76			229 ± 58	0.85	276 ± 18	0.87
	1000			155 ± 25	1.01				
	1500			203 ± 22	1.32				
	2000					177 ± 69	0.65	264 ± 48	0.84
	2500			227 ± 38	1.48				
Proc.fresh3	0	117 ± 9		145 ± 10		237 ± 42		251 ± 8	
	100	90 ± 16	0.77	119 ± 20	0.82	227 ± 29	0.96	244 ± 29	0.97
	500	91 ± 17	0.78	126 ± 9	0.87	156 ± 4	0.66	258 ± 62	1.03
	2000	87 ± 22	0.75	108 ± 35	0.75	243 ± 40	1.02	136 ± 28	0.54
Proc.frozen3	0	146 ± 6		170 ± 28		219 ± 26		241 ± 37	
	25	137 ± 86	0.94						
	50	121 ± 8	0.83						
	75	111 ± 24	0.76						
	100	131 ± 48	0.90	123 ± 41	0.72	200 ± 17	0.82	259 ± 17	1.07
	500	110 ± 15	0.75	123 ± 22	0.72	245 ± 14	1.01	256 ± 21	1.06
	2000			270 ± 25	1.58	263 ± 50	1.08	237 ± 38	0.98
4NQO	5 μg					1219 ± 786			
Sodium azide	10 μg	721 ± 250							
AFB_1	10 μg			769 ± 223				765 ± 211	

(a): Number of Revertants. His^+/plate.

[b] Mutagenic index: no. of His^+ induced in the sample/no. of spontaneous His^+ in the negative control.

* O: Negative control: distilled water; positive control: (−S9) 4-nitroquinoline 1-oxide (4NQO; 5 μg/plate) for TA102 strain and sodium azide (10 μg/plate) for TA100; (+S9) aflatoxin B1 (AFB1: 10 μg/plate) for all strains. Results in bold: positive mutagenics (ANOVA, $p \leqslant 0.05$; dose–response curve, $p \leqslant 0.01$; MI $\geqslant 2$).

Table 4
Summary of the mutagenic activity responses of processed and *in natura* orange juices, for *Salmonella typhimurium* strains without (−S9) and with (+S9) metabolic activation

Juices	*Salmonella typhimurium* Strains							
	TA 98		TA 97a		TA 100		TA 102	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
Org.frozen.	+	+	−	−	−	−	−	−
Org.fresh	+	+	+	+	+	+	−	−
Proc.frozen1	+	+	+	−	−	−	−	−
Proc.fresh1	−	−	−	−	−	−	−	−
Proc.frozen2	+	−	−	−	−	−	−	−
Proc.fresh2	+	−	−	−	−	−	−	−
Proc.frozen3	−	+	−	−	−	−	−	−
Proc.fresh3	−	−	+	−	−	−	−	−

Mutagenic response: + (positive), − (negative).

Table 5
Verification of mutagenicity of *Org.fresh* orange juice

Incubation in presence of orange juice	0 h		3.5 h	
	cfu/ml	$His^+/10^7$ survivors	cfu/ml	$His^+/10^7$ survivors
Control growth	2.9×10^7	2.94 ± 1.01 (17)[a]	9.5×10^7	2.42 ± 0.88 (46)
Org.fresh (+S9) (2000 μl)	3.6×10^7	6.28 ± 3.26 (45)	3.0×10^7	42.3 ± 6.19 (255)

Mutagenicity of orange juice (*Org.fresh*) was determined using *S. typhimurium* strain TA98 (with metabolization). Orange juice, bacteria and buffer were pre-incubated for 0 (control) or 3.5 h in a shaking air-bath at 37 °C. Subsequently, the bacteria were washed with saline buffer, re-suspended in soft agar and plated on agar plates. Results are presented as means ± SD of duplicates. This study was repeated and generated similar data.

[a] Numbers in parentheses represent the sum of His^+ revertants of two plates.

plates, enhance the number of spontaneous his revertants. This possibility was examined for a mutagenic juice sample. Test bacteria were exposed to the juice and either plated immediately or plated after 3.5 h incubation in the presence of the orange juice (Table 5). By determining the survival (c.f.u.) after each treatment (before plating), we could establish that (1) 10% orange juice did not support growth of the bacteria during the 3.5 h exposure (in fact there was a 20% decrease in c.f.u.) and (2) that the juice was mutagenic for strain TA98 (Table 5).

Spearman's correlation coefficient (r_s) shows a strong correlation of phenolic compounds with juice mutagenicity for strains TA97a when metabolically activated ($r_s = 0.833$). The amount of juice-contained vitamin C was also correlated with juice mutagenicity in strain TA97a ($r_s = 0.810$) (data not shown). For all other strains, no correlation between the amount of juice-contained phenolic compounds and of vitamin C was found.

4. Discussion

A positive effect is frequently attributed to the ingestion of fruits and their juices with regard to the prevention of many diseases. It is believed that this protection is partially due to the natural antioxidant substances present in these foods. On the other hand, several antioxidant substances related to natural anti-mutagenicity or anti-carcinogenicity have simultaneously been identified as mutagenic or carcinogenic (Ames & Gold, 1998; Yoshimo, Haneda, Naruse, & Murakami, 1999). Fruits and their juices contain a number of flavonoids and other phenolic compounds. Orange juice is also rich in β-carotene and ascorbic acid and is, therefore, a natural supplier of several antioxidants (Kabasakalis et al., 2000; Wang et al., 1996).

This study shows that orange juices indeed have antioxidant potential, since they could inhibit the hydroxyl radical-caused degradation of deoxyribose (Fig. 1) and also could decrease lipid peroxidation [though only at high concentration (Fig. 3)]. Also, most juices were able to scavenge $O_2^{\cdot -}$ (Fig. 2). Phenolic compounds and vitamin C were identified as possible antioxidants in orange juice (Table 1). Phenolic compounds are able to scavenge radicals and to chelate metals (Halliwell & Gutteridge, 2000; Sanchez-Moreno, Larrauri, & Saura-Calixto, 1999), while vitamin C can play a pro-oxidant role in the presence of transition metals [Fenton reaction (Halliwell, 2001; Sies, Stahl, & Sundquist, 1992)]. These compounds can also act as antioxidants, because of their ability to trap superoxide anions (Sies et al., 1992; Stadler et al., 1994). Depending on the concentrations of

phenolic compounds and of transition metals, a complex can be formed that facilitates the redox process (Khan, Ahmad, & Hadi, 2000; Rice-Evans, Miller, & Paganga, 1997). In general, the phenolic compounds at low concentrations show antioxidant behaviour, while presenting pro-oxidant behaviour at higher concentrations; upon further increasing of their concentration they again show antioxidant behaviour. This always depends on the type (position and number of hydroxyl in the molecule) and the concentration of the phenolic compound, as well on that of the transition metal (Galato, Giacomelli, Ckless, & Spinelli, 1999; Khan et al., 2000). Thus it is possible that, at concentrations up to 1%, the orange juices caused a significant increase of lipid peroxidation, i.e. acted as pro-oxidants while, at 10%, they had antioxidant properties; this effect was most pronounced in fresh juice samples (Fig. 3). Also we wish to point out that the same amount of copper was employed in these tests, whereas the juice concentrations varied by a factor of 10.

The sample with the highest antioxidant effect – *Proc.fresh1* (Figs. 1(b),2(b),3(b)) had a lower concentration of phenolic compounds (Table 1), especially when compared to its frozen form (*Proc.frozen1*). These differences in antioxidant potential may partially be attributed to qualitative variations of the phenolic compounds, as the structural differences may modify the antioxidant potential of phenolic acids (Galato, Ckless, Susin, Giacomelli, Ribeiro-do-Vale, & Spinelli, 2001). Alteration of one or more hydroxyls, or oxidation of the phenolic compounds, may have occurred during the freezing/thawing process, thus leading to a decreased antioxidant potential.

Interestingly, *Proc.fresh2* juice was unable either to trap superoxide anion (Fig. 2(c)) or to decrease the lipid peroxidation at 10% (as compared to 1% concentration) (Fig. 3(c)). This juice is sugar-sweetened, suggesting that the presence of carbohydrates can interfere with the lipid peroxidation assay.

The different results observed for the antioxidant potential of the juices are probably due to various factors, namely climate, soil, fruit variety, degree of maturation (Wang et al., 1996) and to the techniques employed for juice preparation. Juice sample *Proc.fresh1* shows the highest antioxidant activity (Figs. 1–3) and also has no mutagenic potential in the *Salmonella*/microsome test. All other juices, at least in one batch, induced frameshift mutation in TA98 or TA97a. The strongest positive results for mutagenesis were found for *Org.fresh* juice (Table 4). While the different mutagenicity of juices may be explained by the intrinsic differences inherent in the fruits from which they were derived, by variations in processing methods, and to their varying shelf life (Tassara, 1998; Wang et al., 1996), the high mutagenicity of *Org.fresh* juice is not expected to stem from components of the orange peel, as might be expected in industrially processed juices. Amount and quality of phenolic compounds can partially contribute to juice mutagenicity. Moreover, the oxidation of phenolic compounds can generate ROS, partially responsible for the observed mutagenicity (Patrineli, Clifford, Walker, & Ionnides, 1996a; Patrineli et al., 1996b). Thus the mutagenicity of some fresh, frozen and processed *in natura* orange juices may be caused by polyphenol oxidase-generated quinones that are already present in the fruits prior to juice production (Patrineli et al., 1996a, 1996b). These quinones can be converted to semiquinone radicals by loss of one electron, and can directly interact with DNA or facilitate redox cycling. Thus, ROS would be generated in the presence of molecular oxygen, leading to oxidative stress and to DNA damage (Patrineli et al., 1996a, 1996b; Yoshimo et al., 1999).

Processed and *in natura* orange juices contain the phenol quercitin (Hertog, Hollman, & De Putte, 1993). This may explain the induced mutations found in strains TA98 and TA100 (Tables 2 and 3). Caffeic acid is present in citrus fruits and could be another juice component responsible for the detected mutagenicity (Rice-Evans et al., 1997). However, although this monophenolic compound is clastogenic in Chinese hamster ovary (CHO) cells, no mutagenic activity has been observed in strain TA98 (Vargas, Motta, Leitão, & Henriques, 1990).

In our samples, vitamin C alone could not be responsible for the mutagenicity as a pro-oxidant due to the intrinsic presence of a transitional metal in the oranges, since the samples with similar amounts of this vitamin showed different mutagenic responses (see Tables 1 and 4). Therefore, the differences of composition of the oranges from which the juices derived should be held responsible for the different results of mutagenicity. We did not find any food preservatives, e.g., benzoic and sorbic acid or bisulfite (data not shown), and organophosphorous or carbamate pesticides were not detected. However, the influence of pesticides containing transition metals, such as molecular copper, could, in the presence of vitamin C, induce hydroxyl radicals and thus induce mutagenesis (Guecheva, Henriques, & Erdtmann, 2001).

By means of the correlation coefficient of Spearman (r_s), one can clearly see that the mutagenic effect for TA97a, with metabolization ($r_s = 0.833$), is directly related to the presence of total phenolics and also to vitamin C ($r_s = 0.810$) in the juices (data not shown).

In summary, our results show that the analyzed orange juices have antioxidant and mutagenic potential; phenolic compounds and vitamin C can cooperate to enhance the mutagenic activity. However, other antioxidants present in oranges, such as vitamin E, carotenoids and minerals (e.g. selenium), as well as the synergistic effects among all antioxidants (combinations

of different compounds) can increase the total antioxidant potential of the juices, explaining the results observed in the biochemical assays (Halliwell, 2001; Sanchez-Moreno et al., 1999). This strong antioxidant potential of orange juice (Halliwell, 2001; Wang et al., 1996) must be overcome by a solid mutagenic activity in order to induce the observed bacterial mutations. Clearly, it would be interesting to confirm these results using eukaryotic mutagenic test systems (Mavournin, Blakey, Cimino, Salamone, & Heddle, 1990; Tice et al., 2000; Wolf & Peter, 1997).

In spite of the fact that some of the analyzed orange juices had mutagenic activity (Table 2), the risk for humans consuming orange juice may be low, due to enzymatic activities and pH changes in the digestive tract. Also, antimutagenic acting juice ingredients and detoxificants, e.g. carotenoids and some vitamins (Ames, 1989; Halliwell, 2001; Middleton et al., 2000), may prevent damage there.

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