ELSEVIER

Journal of Nutritional Biochemistry 13 (2002) 411–420

THE JOURNAL OF Nutritional Biochemistry

Norbixin ingestion did not induce any detectable DNA breakage in liver and kidney but caused a considerable impairment in plasma glucose levels of rats and mice

Ana C.S. Fernandes[a], Carla A. Almeida[a], Franco Albano[b], Gustavo A.T. Laranja[a], Israel Felzenszwalb[b], Celso L.S. Lage[c], Cristiano C.N.F. de Sa[d], Anibal S. Moura[d], Karla Kovary[a,*,1]

[a]*Departamento de Bioquímica, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil*
[b]*Departamento de Biofísca e Biometria, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil*
[c]*Instituto de Biofísica Carlos Chagas Filho/CCS, Cidade Universitária, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil*
[d]*Departamento de Fisiologia, Instituto de Biologia Roberto Alcantara Gomes, Universidade do Estado do Rio de Janeiro, Rio de Janeiro, Brazil*

Received 8 June 2001; received in revised form 9 January 2002; accepted 5 February 2002

Abstract

From the seeds of *Bixa orellana* are extracted the carotenoids bixin and norbixin that have been widely used for coloring food. In this study, the toxicity of norbixin, purified or not (annatto extract containing 50% norbixin), was investigated in mice and rats after 21 days of ingestion through drinking water. Mice were exposed to doses of 56 and 351 mg/kg (annatto extract) and 0.8, 7.6, 66 and 274 mg/kg (norbixin). Rats were exposed to doses of 0.8, 7.5 and 68 mg/kg (annatto extract) and 0.8, 8.5 and 74 mg/kg (norbixin). In rats, no toxicity was detected by plasma chemistry. In mice, norbixin induced an increase in plasma alanine aminotransferase activity (ALT) while both norbixin and annatto extract induced a decrease in plasma total protein and globulins ($P < 0.05$). However, no signs of toxicity were detected in liver by histopathological analysis. No enhancement in DNA breakage was detected in liver or kidney from mice treated with annatto pigments, as evaluated by the comet assay. Nevertheless, there was a remarkable effect of norbixin on the glycemia of both rodent species. In rats, norbixin induced hyperglycemia that ranged from 26.9% (8.5 mg/kg norbixin, to 52.6% (74 mg/kg norbixin, $P < 0.01$) above control levels. In mice, norbixin induced hypoglycemia that ranged from 14.4% (0.8 mg/kg norbixin, $P < 0.05$) to 21.5% (66 mg/kg norbixin, $P < 0.001$) below control levels. Rats and mice treated with annatto pigments showed hyperinsulinemia and hypoinsulinemia, respectively indicating that pancreatic β-cells were functional. More studies should be performed to fully understand of how species-related differences influences the biological fate of norbixin.

Keywords: Annatto pigments; Norbixin; Comet assay; Plasma glucose; Insulin

1. Introduction

The seeds of *Bixa orellana* L., a native shrub from Tropical America, are a rich source of orange-red pigments that have been largely used by the food coloring industry. These pigments are commercially known as annatto (E160b) and their major coloring component is bixin ($C_{25}H_{30}O_4$), an unusual carotenoid having a free carboxyl and an esterified carboxyl as end groups. Approximately 80% of the pigments present in annatto seeds correspond to bixin [1] and more recently several other minor carotenoids have been isolated and identified [2–4]. The hydrolytic removal of the methyl ester group from bixin by saponification originates norbixin ($C_{24}H_{28}O_4$), a water-soluble carotenoid also found in annatto preparations but in lower amounts than bixin.

Notwithstanding the large use of annatto pigments as food colorant, practically no information exits about their

* Corresponding author. Tel.: +55-21-587-6143; fax: +55-21-587-6136.
E-mail address: kakovary@uerj.br (K. Kovary).
This work was partially supported by FAPERJ, CNPq and SR2/UERJ.
[1] Present address: Instituto de Biofísica Carlos Chagas Filho/CCS, Laboratório de Fisiologia Vegetal, Cidade Universitária, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brasil.

0955-2863/02/$ – see front matter
PII: S0955-2863(02)00177-8

toxicological properties in human and animal tissues. Toxicity was mostly determined in animals using commercial annatto preparations containing undetermined amounts of bixin and norbixin [5,6]. Toxicological data about annatto pigments are limited, possibly because food additives derived from natural sources have been given exemption from certification, i.e. they can be safely used without in-depth chemical and toxicological analysis [7]. Moreover, annatto pigments are not found in dietary fruits and vegetables and hence they are ingested only in tiny amounts as food additives.

Although the use of annatto is not restricted at all in the U.S., there are some legislative restrictions of its consumption in Europe. Currently the allowed daily intake (ADI) for annatto is 0–2.5 mg/kg body weight/day (for a preparation containing 2.6% carotenoids expressed as bixin) and 0–0.065 mg/kg body weight/day expressed as the pure pigment [8]. In contrast, ingestion of annatto pigments in Latin American countries most likely exceeds ADI. In Brazil, the waxy material surrounding annatto seeds is often crushed with corn flour into a farinaceous reddish powder (locally known as *colorau*) and used as a flavoring additive in the Brazilian diet.

Annatto pigments are considered not to be genotoxic based mainly on *in vitro* screening data [9–12]. Most of these data were obtained from bacterial assays (AMES test) and from chromosomal aberration tests performed on human and hamster cells. On the other hand, we recently demonstrated that fibroblasts treated with norbixin *in vitro* were rendered either resistant or susceptible to DNA damage induced by hydrogen peroxide, as measured by the comet assay [13]. DNA integrity was subordinated to norbixin concentration, with low amounts favoring DNA protection while concentrations above 50 μM preferentially enhanced oxidant-induced DNA damage. The detected prooxidant activity of norbixin is in fact an undesirable biological characteristic that has been recognized to be intrinsically associated with many other carotenoids including β-carotene. Depending on their redox potential as well as on the biological environment in which they act, carotenoids can have their healthy antioxidant activity shifted to a pathologic prooxidant activity. The effectiveness of this prooxidant activity is conditioned to several factors such as oxygen tension, carotenoid concentration, and interaction with other antioxidants [14].

We decided to investigate in rats and mice the toxicity of annatto pigments. Considering that little is known about the metabolism of annatto pigments, animals were treated for 21 days, a sufficient time for a potential accumulation of the pigments. Two preparations of annatto pigments were tested: purified norbixin and a saponified annatto extract containing 50% norbixin. Both preparations were administrated daily to the animals in drinking water so that the exposure doses could be easily controlled. The chemical toxicity of the annatto pigments was investigated by evaluating selected biochemical markers of hepatic and renal functions. Plasma levels of triglycerides, total cholesterol and glucose were also determined as indicators of metabolic functionality. The *in vivo* genotoxic potential of the annatto pigments was also investigated by determining the frequency of DNA breakage in liver and kidney of experimental mice by the comet assay.

2. Materials and methods

2.1. Annatto pigments

Purification of bixin from annatto seeds and its conversion to norbixin by saponification have been described elsewhere [13]. The purity of norbixin was higher than 98% based on high-performance liquid chromatography (HPLC) analysis. An annatto extract containing 50% bixin along with many other unidentified molecules was prepared by extracting annatto seeds three times with ethanol:water (93:7, v/v) with vigorous shaking at 37°C for several hours. Combined alcoholic extracts were filtered through filter paper to remove a fraction of bixin rendered insoluble by the extraction solution. The alcoholic extract was concentrated in a rotary evaporator at 37°C followed by washing of the oily leftover material with hexane by stirring. Residual hexane was evaporated at 50°C and the resulting annatto pigments were rendered water-soluble by saponification in aqueous NaOH solution for several hours at 37°C. Water was evaporated to dryness at 50°C and dehydrated pigments were kept at −20°C until further use. All other reagents used were of the highest purity and were purchased from Sigma (St. Louis, MO), Merck (Rio de Janeiro, Brazil) and GibcoBRL (Gaithersburg, MD).

2.2. Animal treatment with annatto pigments

Three-month-old male Swiss mice (average weight 35–45 g) and two-month-old female Wistar rats (average weight 185–195 g) were purchased from a local laboratory animal supplier (Rio de Janeiro, Brazil). Animals were housed with free access to food and water for at least one week prior to the experiments. Rats and mice were fed *ad libitum* with a commercial standard rodent diet (Nuvilab Ltd, Curitiba, Brazil) and had free access to drinking water consisting of tap water alone (control animals) or tap water containing annatto pigments (experimental animals). Mice were treated with two doses of annatto extract (group 1, 26.5 mg%, and group 2, 133.0 mg%) or three doses of purified norbixin (group 3, 0.3 mg%; group 4, 2.7 mg%; and group 5, 26.5 mg%). Rats were treated with three doses of annatto extract (groups 1 to 3) or three doses of norbixin (groups 4 to 6). Doses tested in rats were: groups 1 and 4, 0.6 mg%; groups 2 and 5, 5.7 mg%, and groups 3 and 6, 56.7 mg%. The solutions of drinking water containing annatto pigments were always prepared freshly and changed every day. The volume of fluid drunk by the animals was

recorded daily in order to calculate the average amount of annatto pigments ingested per animal per day. After 21 days of treatment, animals were lightly anaesthetized with ether and sacrificed by cardiac puncture. Blood was collected from all treated and untreated animals using the same method and time of blood collection was the same throughout the study (early in the morning) to avoid diurnal variations on plasma chemistry. Animals were not fasted before blood collection. Separation of plasma from blood cells was always performed soon after blood collection by centrifuging heparinized blood at 5,000 rpm for 5 min and kept at −20°C for further biochemical determinations.

2.3. Biochemical determinations

Plasma samples were analyzed for glucose, total cholesterol, triglycerides, urea, creatinine, total protein, albumin, and globulins (Mega bioanalyzer, Merck), and for the enzymatic activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) (Cobas Integra bioanalyzer, Roche), using appropriate kits supplied by the manufacturers. Immunoreactive plasma insulin was measured by radioimmunoassay [15] using ^{125}I-labeled porcine insulin (Amersham, USA) as a tracer, anti-insulin antibody from guinea pig (kindly provided by Dr. William Mallaisse, Brussels, Belgium) and purified rat insulin (Novo Nordisk, New York, NY, USA) as standard. The plasma of each control and experimental animal was evaluated individually.

2.4. Single-cell gel electrophoresis (or comet) assay

Cells from liver and kidney were dissociated by mincing each organ into very fine fragments in ice-cold dissociation solution (Ca^{2+} and Mg^{2+} free Hanks Balanced Salt Solution supplemented with 20 mM EDTA). Minced tissues were left to settle for a couple of minutes before the comet assay in order to sediment the larger debris. The alkaline version of the comet assay, which detects single- and double-stranded DNA breaks, was performed according to the method originally described by Singh et al. [16] with minor modifications. Briefly, 10 μL of liver or kidney cell suspension were mixed with 120 μL of 0.5% low-melting-temperature agarose in phosphate-buffered saline (PBS) and added to microscope slides precoated with 1.5% normal-melting-temperature agarose in PBS. Slides were covered with a microscope coverslip and refrigerated for 5 min to gel, followed by immersion in ice-cold alkaline lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% DMSO, 1% Triton X-100, final pH >10.0) for at least 1 h. Slides were then incubated for 20 min in ice-cold electrophoresis solution (0.2 M NaOH, 1 mM EDTA), followed by electrophoresis at 25 V/300 mA for 25 min. After electrophoresis, slides were rinsed with water, allowed to dry at 37°C and stained for five minutes with 20 μg/mL ethidium bromide. The DNA of individual cells was viewed using an epifluorescence microscope (Olympus) with 516–560 nm emission from a 50 W mercury light source. DNA breakage was quantitated by visual scoring of 50 randomly selected cells per slide, which were classified into five categories, each representing each a different degree of damage, ranging from no comet (type 0, no damaged cells) to maximum length comet (type 4, highly damaged cells). Comets of type 1 are representative of cells with a minimal detectable frequency of DNA lesions (very low damage), while comets of types 2 and 3 are representative of cells with a moderately low to moderately high frequency of DNA lesions, respectively. The slides were analyzed by investigators blinded to the experimental conditions used for the treatment of the mice from which the organ samples were prepared. A score of total damage was arbitrarily assigned to each treatment by multiplying the number of cells allocated to each category of DNA damage by the numerical value of the corresponding category and summing all categories, yielding a maximum possible score of 200.

2.5. Statistical analysis

All animal data are expressed as mean ± SEM (standard error of the mean) and were analyzed statistically by one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparisons test using GraphPad InStat program (GraphPad Software, Inc.). Differences were considered statistically significant at a P-value of less than 0.05.

3. Results

3.1. Influence of annatto pigments on growth parameters

Selection of the doses were based on the ADI for norbixin (0.065 mg/kg) being the lowest dose tested approximately 10 times this value while the subsequently doses ranged on a 5–10 fold scale. Based on the average daily fluid intake, the following exposure doses were tested in mice: 56 and 351 mg/kg for the annatto extract (groups 1 and 2) and 0.8, 7.6, 66 and 274 mg/kg for norbixin (groups 3 to 6, respectively) (Table 1). Exposure doses for rats were 0.8, 7.5 and 67 mg/kg for annatto extract (groups 1 to 3), and 0.8, 8.5 and 74 mg/kg for norbixin (groups 4 to 6) (Table 2). No considerable variation in weight gain was observed in mice and rats treated with annatto extract or purified norbixin (Tables 1 and 2, respectively). A small increase in the weights of liver (10.9%) and kidney (12.4%) of mice treated with the highest dose of norbixin (group 6, 274 mg/kg) was detected but without reaching statistical significance (Table 1). No significant variations were observed in the final organ weights (liver and kidney) of experimental rats (Table 2).

Table 1
Influence of annatto pigments on body, liver and kidney weights of male Swiss mice

Treatment	Average daily liquid intake (mL)	Average daily exposure dose (mg/kg)	Body weight (g)			Organ weight (g/100 g body weight)	
			Initial	Final	Net gain	Liver	Kidney (both)
Control ($n = 17$)	10.3 ± 0.5	—	36.8 ± 1.5	41.5 ± 1.8	4.7	5.40 ± 0.14	1.29 ± 0.04
Annato extract:							
Group 1 ($n = 7$)	9.2 ± 0.3	56	41.2 ± 1.2	46.0 ± 1.2	4.8	5.30 ± 0.22	1.30 ± 0.04
Group 2 ($n = 6$)	10.6 ± 0.3	351	38.5 ± 0.7	42.1 ± 1.5	3.6	5.58 ± 0.29	1.33 ± 0.05
Norbixin							
Group 3 ($n = 10$)	10.5 ± 0.3	0.8	38.0 ± 0.9	41.5 ± 1.1	3.5	5.27 ± 0.09	1.35 ± 0.03
Group 4 ($n = 16$)	10.6 ± 0.3	7.6	36.0 ± 0.9	39.4 ± 1.2	3.4	5.11 ± 0.11	1.28 ± 0.03
Group 5 ($n = 17$)	10.1 ± 0.3	66	38.7 ± 1.0	42.5 ± 1.1	3.8	5.25 ± 0.14	1.29 ± 0.05
Group 6 ($n = 7$)	9.5 ± 0.3	274	43.9 ± 0.6	48.4 ± 0.8	4.5	5.99 ± 0.23	1.45 ± 0.10

Data are expressed as mean ± SEM from two to three independent experiments, with the exception of groups 2 and 6 whose data were compiled from a single experiment each. *n*, number of individual mice analyzed.

3.2. Plasma indicators of liver pathology in mice and rats treated with annatto pigments

Plasma ALT, AST and ALP were determined since an increase in their activities is frequently associated with liver toxicity (Tables 3 and 4). In mice treated with annatto pigments, plasma activities of AST and ALP were all within the normal ranges observed in control mice. Similarly, no significant variation in plasma ALT was detected in mice treated with annatto extract (Table 3, groups 1 and 2), whereas mice treated with norbixin showed a dose-related increase in plasma ALT that ranged from 70% in group 3, 134% in group 4 and 158% in group 5. However, the variation observed among means was not significantly greater than expected by change. In contrast, mice from group 6 (highest dose of norbixin) showed values of plasma ALT comparable to those found in control mice. No substantial differences could be detected in plasma activities of any of the standard enzymes investigated in rats treated either with annatto extract or with purified norbixin (Table 4).

The levels of plasma proteins (total protein, albumin, and globulins) were also determined since they are useful markers of hepatic synthetic functionality. A dose-related decrease in total protein was detected in mice treated either with annatto extract or with norbixin (Table 3) but this decrease was significant only in mice from group 2 (annatto extract, 351 mg/kg, $P < 0.05$). With the exception of mice from group 3 (0.8 mg/kg of norbixin), all other experimental groups showed some reduction in plasma levels of globulins, which was only significant in mice from group 2 (22.6%, $P < 0.05$). Although mice from group 6 did not show any significant alteration in total plasma protein, there was still a reduction in plasma globulins (though not statistically significant). On the other hand, no significant variations could be detected in plasma levels of total protein, albumin or globulins in rats treated either with annatto extract or with norbixin when compared to control rats (Table 4).

3.3. Plasma indicators of kidney functionality in mice and rats treated with annatto pigments

Renal functionality was monitored by determining plasma levels of urea and creatinine and the values found for mice and rats are shown in Tables 3 and 4, respectively.

Table 2
Influence of annatto pigments on body, liver and kidney weights of female Wistar rats

Treatment	Average daily liquid intake (mL)	Average exposure dose (mg/kg/day)	Body weight (g)			Organ weight (g/100 g body weight)	
			Initial	Final	Net gain	Liver	Kidney (both)
Control ($n = 15$)	26.4 ± 1.3	—	195 ± 4	205 ± 4	10	3.47 ± 0.11	0.64 ± 0.01
Annatto extract:							
Group 1 ($n = 5$)	25.3 ± 0.5	0.8	195 ± 10	204 ± 9	9	3.44 ± 0.12	0.61 ± 0.01
Group 2 ($n = 5$)	25.6 ± 0.6	7.5	189 ± 9	201 ± 10	12	3.35 ± 0.09	0.62 ± 0.02
Group 3 ($n = 5$)	23.3 ± 0.5	68	189 ± 6	202 ± 6	13	3.68 ± 0.22	0.64 ± 0.02
Norbixin:							
Group 4 ($n = 5$)	28.0 ± 1.5	0.8	192 ± 4	203 ± 8	11	3.24 ± 0.13	ND
Group 5 ($n = 5$)	30.3 ± 1.9	8.5	196 ± 11	209 ± 9	13	3.50 ± 0.10	ND
Group 6 ($n = 5$)	25.8 ± 1.1	74	193 ± 3	202 ± 6	9	3.33 ± 0.07	ND

Data are expressed as mean ± SEM from one experiment. *n*, number of individual rats analyzed.
ND = Not determined.

Table 3
Clinical plasma parameters of male Swiss mice after ingestion of annatto pigments

Parameter	Control mice	Treatment[a]					
		Annatto extract		Norbixin			
		Group 1 (56 mg/kg)	Group 2 (351 mg/kg)	Group 3 (0.8 mg/kg)	Group 4 (7.6 mg/kg)	Group 5 (66 mg/kg)	Group 6 (274 mg/kg)
Plasma enzymes (IU/L, 37°C):							
AST	208 ± 25 (17)	214 ± 6 (7)	222 ± 30 (6)	195 ± 49 (8)	216 ± 46 (10)	245 ± 40 (11)	268 ± 54 (7)
ALT	70 ± 9 (21)	47 ± 4 (7)	49 ± 3 (6)	119 ± 29 (7)	164 ± 66 (9)	179 ± 62 (11)	63 ± 8 (7)
ALP	85 ± 7 (18)	106 ± 9 (6)	99 ± 8 (6)	101 ± 17 (6)	103 ± 11 (8)	106 ± 16 (4)	ND
Plasma proteins:							
Total protein (g/dL)	4.71 ± 0.09 (21)	4.36 ± 0.07 (7)	4.25 ± 0.10 (6)	4.66 ± 0.30 (8)	4.44 ± 0.11 (10)	4.33 ± 0.08 (11)	4.74 ± 0.13 (7)
Albumin (g/dL)	2.36 ± 0.09 (21)	2.37 ± 0.06 (7)	2.43 ± 0.08 (6)	2.24 ± 0.11 (8)	2.35 ± 0.06 (10)	2.17 ± 0.16 (11)	2.65 ± 0.06 (7)
Globulins (g/dL)	2.35 ± 0.11 (21)	1.99 ± 0.03* (7)	1.82 ± 0.04* (6)	2.42 ± 0.27 (8)	2.09 ± 0.08* (10)	2.16 ± 0.12 (11)	2.09 ± 0.07 (7)
Plasma metabolites:							
Urea nitrogen (mg/dL)	58 ± 2 (17)	58 ± 3 (7)	52 ± 4 (6)	57 ± 4 (8)	54 ± 2 (10)	52 ± 2* (11)	53 ± 3 (7)
Creatinine (mg/dL)	0.30 ± 0.02 (21)	0.27 ± 0.02 (7)	0.25 ± 0.02 (6)	0.26 ± 0.03 (8)	0.26 ± 0.02 (10)	0.19 ± 0.01** (10)	0.26 ± 0.02 (7)
Glucose (mg/dL)	195 ± 6 (21)	194 ± 6 (7)	157 ± 10** (10)	167 ± 8* (13)	156 ± 7.0*** (16)	153 ± 6*** (16)	201 ± 7 (7)
Cholesterol (mg/dL)	78 ± 3 (21)	71 ± 4 (7)	75 ± 4 (10)	77 ± 5 (13)	71 ± 3 (15)	71 ± 4 (15)	78 ± 5 (7)
Triglycerides (mg/dL)	163 ± 15 (21)	136 ± 16 (7)	105 ± 17 (6)	135 ± 12 (8)	169 ± 11 (10)	174 ± 12 (10)	136 ± 20 (7)

[a] Number in parenthesis correspond to the total number of independent mice analyzed. Data are expressed as mean ± SEM from two to three independent experiments, with exception of group 6 whose result were compiled from a single experiment. ND, not determined.

* $P < 0.05$ or ** $P < 0.01$ or *** $P < 0.001$ versus control mice.

Table 4
Clinical plasma parameters of female Wistar rats after three weeks ingestion of annatto pigments

Parameter	Control rats ($n = 15$)	Treatment[a]					
		Annatto extract			Norbixin		
		Group 1 (0.8 mg/kg)[b] $n = 5$	Group 2 (7.5 mg/Kkg) $n = 5$	Group 3 (68 mg/kg) $n = 5$	Group 4 (0.8 mg/kg) $n = 5$	Group 5 (8.5 mg/kg) $n = 5$	Group 6 (74 mg/kg) $n = 5$
Plasma enzymes (IU/L, 37°C):							
AST	126 ± 19	104 ± 2	154 ± 45	103 ± 8	96 ± 8	91 ± 8	87 ± 12
ALT	59 ± 5	64 ± 4	59 ± 4	62 ± 3	44 ± 3	50 ± 2	43 ± 2
ALP	102 ± 7	101 ± 11	94 ± 12	92 ± 11	112 ± 8	107 ± 9	75 ± 4
Plasma proteins:							
Total protein (g/dL)	6.01 ± 0.18	6.40 ± 0.11	6.34 ± 0.14	6.30 ± 0.04	6.48 ± 0.15	6.62 ± 0.16	6.56 ± 0.09
Albumin (g/dL)	2.66 ± 0.10	2.74 ± 0.11	2.78 ± 0.09	2.90 ± 0.05	2.68 ± 0.16	3.02 ± 0.06	2.86 ± 0.03
Globulins (g/dL)	3.35 ± 0.15	3.66 ± 0.07	3.56 ± 0.09	3.40 ± 0.05	3.80 ± 0.05	3.60 ± 0.13	3.70 ± 0.07
Plasma metabolites:							
Urea nitrogen (mg/dL)	49 ± 4	48 ± 2	54 ± 1	51 ± 4	53 ± 8	50 ± 2	40 ± 1
Creatinine (mg/dL)	0.44 ± 0.02	0.48 ± 0.02	0.44 ± 0.03	0.41 ± 0.01	0.46 ± 0.03	0.46 ± 0.02	0.46 ± 0.03
Glucose (mg/dL)	78 ± 5	100 ± 4	103 ± 6*	111 ± 7**	83 ± 8	99 ± 3	119 ± 8**
Total cholesterol (mg/dL)	57 ± 4	43 ± 3	39 ± 3*	67 ± 4	54 ± 4	61 ± 4	56 ± 4
Triglycerides (mg/dL)	50 ± 9	58 ± 8	57 ± 10	72 ± 6	71 ± 8	71 ± 14	56 ± 5

[a] Data are expressed as mean ± SEM of a single experiment. n, number of individual rats analyzed.
[b] Average exposure dose (mg/kg/day).
* $P < 0.05$ or ** $P < 0.01$ versus control rats.

Basically all experimental mice showed a slight reduction in plasma urea when compared to control mice (Table 3). Plasma creatinine was also reduced in mice treated with annatto pigments being statistically significant only in mice from group 5 (36.7% of reduction, $P < 0.01$). Decreased levels of creatinine were also detected in the other experimental groups but without reaching statistical significance (Table 3). In rats treated with annatto pigments, no significant alterations were observed in plasma levels of urea and creatinine when compared with their respective control levels (Table 4).

3.4. Plasma metabolites in mice and rats treated with annatto pigments

Plasma levels of triglycerides, total cholesterol and glucose were investigated as indicators of the metabolic status of the animals (Tables 3 and 4). Mice treated with annatto extract or with norbixin did not show any significant alteration in plasma triglycerides or in plasma total cholesterol when compared to control mice (Table 3). Similarly, rats treated with annatto pigments did not show any significant variation in plasma triglycerides (Table 4). Total cholesterol was reduced in rats treated with annatto extract, with reductions of 24.6% (group 1, not statistically significant) and 31.6% (group 2, $P < 0.05$) (Table 4). Rats treated with norbixin did not exhibit any considerable variation in plasma levels of total cholesterol.

Plasma levels of glucose were significantly reduced in mice treated with annatto pigments (Table 3). This reduction reached 19.5% (group 2, $P < 0.01$) in mice treated with annatto extract while in mice treated with norbixin, these reductions were of 14.4% (group 3, $P < 0.05$), 20% (group 4, $P < 0.001$), and 21.5% (group 5, $P < 0.001$). On the other hand, mice from group 6 did not show alteration in plasma glucose, whose levels were quite similar to control levels. In contrast, rats treated with annatto pigments showed a dose-related increase rather than a decrease in plasma glucose (Table 4). In rats treated with annatto extract, the increase in plasma glucose was of 28.2% in group 1, 32% in group 2 ($P < 0.05$) and 42.3% in group 3 ($P < 0.01$), while in rats treated with norbixin, this increase was of 6.4% in group 4, 26.9% in group 5 (not statistically significant) and 52.6% in group 6 ($P < 0.01$).

3.5. Plasma levels of insulin in mice and rats treated with annatto pigments

As annatto pigments induced hypoglycemia in mice and hyperglycemia in rats, plasma insulin levels were investigates in both rodent species and the results obtained are shown in Fig. 1. In mice treated with norbixin up to 66 mg/kg, there was a concomitant decrease in plasma insulin. Even under conditions of apparently normal glycemia (274 mg/kg of norbixin), plasma levels of insulin were below control levels (Table 3 and Fig. 1A).

In rats treated with norbixin up to 8.5 mg/kg, the resulting hyperglycemia was followed by an increase in plasma insulin (Fig. 1B). Higher doses of norbixin (up to 74 mg/kg), which induced the highest level of glycemia, resulted in

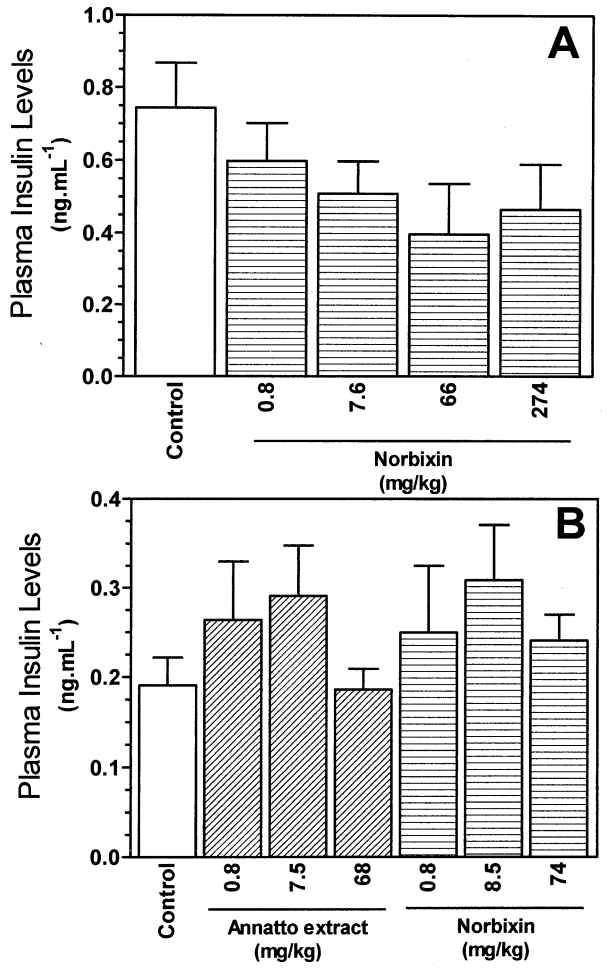

Fig. 1. Insulin levels in plasma of mice (A) and rats (B) after 21 days of ingestion of norbixin or annatto extract through drinking water.

insulin levels that were similar to those observed in control rats. Similar variations in plasma insulin were also observed in rats that ingested a dose of annatto extract up to 68 mg/kg (Fig. 1B).

3.6. Evaluation of DNA integrity in liver and kidney cells from mice treated with annatto pigments

To verify if annatto extract or norbixin was genotoxic to mice, DNA from liver and kidney was examined by the comet assay for the presence of single and double strand breaks and the results obtained are shown in Tables 5 and 6, respectively. No genotoxic effect could be detected in either organ, as demonstrated by the absence of a significant increase in the frequency of DNA strand breaks above control levels.

4. Discussion

Liver and kidney were the major organs investigated in mice and rats treated with annatto pigments, as their functional integrity is vital for whole body homeostasis. Liver seems to be the major site where annatto pigments are metabolized [17], this being a pivotal organ in the metabolism of many other carotenoids [18]. Nevertheless, it is unknown by which means annatto pigments are absorbed in the gastrointestinal tract or if the vehicle of administration (liquid versus solid) influences the absorption efficiency. HPLC analyses of human plasma after ingestion of a single dose of annatto food color indicated that there is a significant conversion of bixin to norbixin in the intestinal tract and in the bloodstream [19]. Studies about the effects of different carotenoids on xenobiotic metabolizing enzymes performed on rats indicated that bixin accumulates primarily in the small intestine at very high levels [20]. Significant amounts of bixin were also detected in liver, lung and kidney but if the ingested bixin was partially converted to norbixin, as observed in man, was not evaluated by the authors.

No obvious toxicity associated with annatto extract or norbixin was detected in rats, as shown by body and organ (liver and kidneys) weights and plasma chemistry (enzymes, plasma proteins, urea and creatinine) (Tables 2 and

Table 5
Effect of the cumulative ingestion of annatto extract or purified norbixin on DNA integrity of mouse liver cells

Treatment	Frequency of DNA strands breaks					Total score (out of 200)
	No damage (type 0)	Very low (type 1)	Moderately low (type 2)	Moderately high (type 3)	Very high (type 4)	
Control mice (n = 10)	41.8 ± 1.5	5.7 ± 0.9	1.0 ± 0.5	0.5 ± 0.3	1.0 ± 0.4	13.2 ± 3.0
Annatto extract:						
Dose 1 (56 mg/kg/day) (n = 5)	41.2 ± 1.7	5.6 ± 1.3	1.6 ± 0.5	0.8 ± 0.4	0.8 ± 0.4	14.4 ± 2.8
Dose 2 (351 mg/kg/day) (n = 5)	42.0 ± 0.3	5.4 ± 0.5	1.2 ± 0.4	1.0 ± 0.3	0.4 ± 0.4	12.4 ± 1.1
Norbixin:						
Dose 1 (0.8 mg/kg/day) (n = 10)	41.1 ± 1.1	4.9 ± 0.5	0.9 ± 0.3	1.0 ± 0.3	2.4 ± 0.5	18.1 ± 3.3
Dose 2 (7.6 mg/kg/day) (n = 10)	42.4 ± 1.1	5.2 ± 0.9	0.6 ± 0.2	1.4 ± 0.3	0.4 ± 0.2	12.2 ± 1.5
Dose 3 (66 mg/kg/day) (n = 10)	46.0 ± 0.5	2.4 ± 0.3	0.7 ± 0.2	0.4 ± 0.2	0.4 ± 0.3	19.4 ± 6.2
Dose 4 (274 mg/kg/day) (n = 10)	42.2 ± 1.2	4.2 ± 1.1	2.0 ± 0.5	0.6 ± 0.4	1.2 ± 0.5	14.4 ± 2.4

Total score was calculated as described in Materials and Methods. Each result corresponds to the mean ± SEM.
n, number of individual mice analyzed.

Table 6
Effect of the cumulative ingestion of annatto extract or purified norbixin on DNA integrity of mouse kidney cells

Treatment	Frequency of DNA strand breaks					Total score (out of 200)
	No damage (type 0)	Very low (type 1)	Moderately low (type 2)	Moderately high (type 3)	Very high (type 4)	
Control mice ($n = 10$)	39.2 ± 1.2	6.4 ± 0.7	1.4 ± 0.3	0.6 ± 0.3	2.3 ± 0.3	20.2 ± 2.4
Annatto extract:						
Dose 1 (56 mg/kg/day) ($n = 5$)	44.2 ± 1.4	3.0 ± 0.3	1.6 ± 0.6	0.0 ± 0.0	1.2 ± 1.0	11.0 ± 4.6
Dose 2 (351 mg/kg/day) ($n = 5$)	32.8 ± 4.0	13.2 ± 2.8	1.4 ± 0.6	0.8 ± 0.4	1.8 ± 0.7	26.4 ± 7.7
Norbixin:						
Dose 1 (0.8 mg/kg/day) ($n = 10$)	34.1 ± 1.8	10.8 ± 1.3	2.3 ± 0.8	1.4 ± 0.8	1.3 ± 0.2	25.8 ± 2.8
Dose 2 (7.6 mg/kg/day) ($n = 10$)	37.1 ± 1.2	6.8 ± 0.3	1.6 ± 0.2	1.1 ± 0.3	3.4 ± 1.0	26.9 ± 4.4
Dose 3 (66 mg/kg/day) ($n = 10$)	40.0 ± 1.5	4.8 ± 0.4	0.9 ± 0.2	1.1 ± 0.4	3.2 ± 1.2	22.7 ± 5.5
Dose 4 (274 mg/kg/day) ($n = 5$)	41.8 ± 3.0	4.2 ± 1.8	1.2 ± 0.4	1.2 ± 0.8	1.6 ± 0.7	15.8 ± 6.7

Total score was calculated as described in Materials and Methods. Each result corresponds to the mean ± SEM.
n, number of individual mice analyzed.

4). Moreover, no genotoxic effect could be detected when DNA of blood cells were evaluated by the comet assay (data not shown).

Nevertheless, mice treated with norbixin showed higher levels of plasma ALT than control mice (Table 3). Although no significant statistical difference among means has been demonstrated, a difference from control was noted in three dose groups and the incidence increased as the dose level of norbixin increased (with exception of the highest dose tested). This indicates that the increase in plasma ALT was most likely a treatment-related effect. An increase in plasma ALT activity usually indicates cellular leakage and loss of functional integrity of hepatic cellular membranes, as this aminotransferase is highly specific for liver [21]. Histopathological analysis by light microscopy and hematoxylin-eosin staining of livers from these mice, however, did not reveal any gross abnormality of hepatic lobular architecture when compared to livers from control mice (data not shown). In addition, results from the comet assay indicated that norbixin is not cytotoxic to liver cells, otherwise we would have detected a particular increase in the frequency of comet type four, whose DNA breakage pattern is indicative of cell death (Table 5). In addition, no increase in the activity of plasma AST has been detected. Usually, plasma ALT and AST levels are equally elevated in cases of acute hepatic necrosis. Therefore, the increase in plasma ALT detected in mice treated with norbixin may be of no significance or, at least, its significance must be determined considering other factors. The comet assay also demonstrated that norbixin and/or some other substances present in annatto extract are not genotoxic to liver and kidney since no enhancement in overall DNA breakage frequency was observed even after ingestion of a dose of norbixin that was more than 4,000 times higher than its ADI (group 6, 274 mg/kg) (Tables 5 and 6). Recently, the alkaline version of the comet assay has been recommended by the International Workshop on genotoxicity test procedures as the optimal version for the identification of agents with genotoxic activity [22].

The ingestion of norbixin up to 66 mg/kg by mice caused a small but persistent reduction in plasma levels of total protein and globulins while albumin levels were within normal ranges (Table 3). Reduced plasma levels of total protein and globulins were also detected in mice treated with annatto extract but the levels of plasma ALT were normal. The observed decrease in total protein levels may have been due to an overall reduction in hepatic protein synthesis since albumin and most alpha and beta proteins are synthesized in the liver. Low plasma total protein concentration was not reflected in the circulating levels of albumin because its plasma half-life is approximately 20 days.

In mice treated with annatto pigments, no expressive alterations were observed in the levels of plasma urea while plasma creatinine was reduced, particularly in mice treated with norbixin up to 66 mg/kg (Table 3). Urea and creatinine are excreted in the urine and renal dysfunction is usually associated to increased plasma concentration of both substances. Creatinine is a waste product mostly derived from endogenous sources particularly from muscle mass and its plasma concentration usually is not affected by extrarenal factors.

In contrast, the continuous ingestion of annatto extract or norbixin by the animals caused hyperglycemia in rats and hypoglycemia in mice (Tables 3 and 4, respectively). Morrison *et al*. [23–25] had already determined that annatto seed coat contains substances capable of altering plasma glucose levels in dogs. The response in dogs observed hyper- and hypo-glycemia, depending on the extraction method from the annatto seed coat. The trans-bixin was determined to be the active component that produced hyperglycemia but only in undernourished conditions. The effect was reversed with riboflavin addition to the diet [25]. The hyperglycemic effect of norbixin in rats apparently was not a consequence of liver damage since no signs of hepatotoxicity were detected. Type β-cells of the pancreatic islets were also functional as there was a tendency to a parallel increase in plasma insulin levels when rats were treated with norbixin up to 8.5 mg/kg

or annatto extract up to 7.5 mg/kg (Fig. 1A). At higher exposure doses, hyperglycemia was associated with normoinsulinemia, suggesting a desensitization of β-cells in their fuel-sensing system.

The effects of annatto pigments in glucose homeostasis of rats might be due to an interaction of norbixin with plasma membranes that could have resulted in altered membrane permeability to glucose, i.e. by affecting GLUT receptor intrinsic activity and/or turnover, causing a lower glucose uptake and consequently a lasting hyperglycemia. On the other hand, it might be that norbixin affected insulin responsiveness by interfering, for example, with insulin receptor activity due to a change in membrane fluidity. It is well documented that polar carotenoids have the ability to interact with lipids in lipid bilayer membranes in a way that affects their biological properties, as demonstrated in plants [26–28], photosynthetic bacteria [29] and artificial membranes [30–32]. Changes in the physicochemical properties of membranes interfere with their phase transition, fluidity and permeability and therefore the regulation of membrane-embedded receptors as well as signal transduction pathways and nutrient transport might be affected to some degree [33]. In mammals, fewer studies have been performed on the effects of polar carotenoids on cell membranes. An exception is the macular membrane of human retina, which contains very high concentrations of zeaxanthin and lutein that appear to play an important role in protecting the macula against age-related degeneration [34].

In contrast to the results observed in rats, norbixin induced a low but highly reproductive and persistent hypoglycemia in mice (Table 3). Since there was a concomitant decrease in plasma insulin levels (Fig. 1B), hypoglycemia was not the consequence of a hyperinsulinemic effect. Hypoglycemia might be also justified by altered membrane fluidity that led to increased glucose uptake due to an increase in the affinity and/or a decrease in the turnover of GLUT receptors. Accumulation of norbixin on hepatic membranes may have also interfered with the secretion of plasma proteins and/or cytosolic retention of ALT in mice. However, if the effects of norbixin are mainly due to interaction with membranes affecting their functionality, then rats and mice show quite different membrane sensitivity to norbixin action. This cannot be explained based simply on membrane dynamics unless rat and mouse membranes are differentially targeted by norbixin, i.e. peripheral tissues (muscle and adipose tissue) versus liver. It might be that the hepatic membranes of mice are more sensitive to a norbixin interaction than rat liver membranes but further experiments are required to evaluate all these questions.

On the other hand, mice that received the highest dose of norbixin (274 mg/kg) showed normoglycemia and plasma total protein and ALT activity within normal ranges. Considering that the process of compound absorption and disposition can be capacity-limited (i.e. saturable) it is possible that at high doses, the absorption, metabolization and route of excretion of norbixin as well as its solubility may be significantly different from that seen at lower doses as otherwise quiescent secondary routes of metabolism become activated [35,36]. Mice that received 351 mg/kg of annatto extract, which corresponds to 175 mg/kg of norbixin, showed overall alterations similar to those detected in mice treated with norbixin up to 66 mg/kg.

The differences in responses observed in mice and rats suggest that differential sensitiveness to annatto pigments may exist among different species with rats being more resistant than mice to a disruption in homeostasis except for glucose metabolism. Since we used female rats and male mice in our study it could be that gender-related differences may have also influenced sensitivity to annatto pigments in these species, and perhaps in man, as recognized to occur to many other substances [37,38]. Understanding the dynamics of norbixin interaction with membranes may help to elucidate how glucose homeostasis is affected by this carotenoid and perhaps by some other polar carotenoids more commonly found in dietary vegetables and fruits.

Acknowledgments

We thank Dr. Paranaguá dos Santos Moreira, Department of Pathology and Laboratories (UERJ/Faculty of Medical Sciences), for the histophatological analysis of the livers, and Luciano M. Alves for excellent technical assistance.

References

[1] H.D. Preston, M.D. Rickard, Extraction and chemistry of annatto, Food Chem 5 (1980) 47–56.
[2] A.Z. Mercadante, A. Steck, H. Pfander, Isolation and structure elucidation of minor carotenoids from annatto (Bixa orellana L.) seeds, Phytochemistry 46 (1997) 1379–1383.
[3] A.Z. Mercadante, A. Steck, H. Pfander, Isolation and identification of new apocarotenoids from annatto (Bixa orellana) seeds, J Agric Food Chem 45 (1997) 1050–1054.
[4] A.Z. Mercadante, A. Steck, H. Pfander, Three minor carotenoids from annatto (Bixa orellana) seeds, Phytochemistry 52 (1999) 135–139.
[5] J. Engelberth-Holm, S. Iversen, Is the vegetable annatto butter colour cancerogenic? Acta Pathol Mic Sc 37 (1955) 483–492.
[6] G.J. van Esch, H. van Genderen, H.H. Vink, Über die chronische Vertraglichkeit von Annattofarbstoff. Zeitschrift für Lebensmitteleuntersuchung und Forschung 111 (1959) 93–108.
[7] J.B. Hallagan, D.C. Allen, J.F. Borelleca, The safety and regulatory status of food, drug and cosmetics colour additives exempt from certification, Food Chem Toxicol 33 (1995) 515–528.
[8] JECFA (1982), Evaluation of certain food additives and contaminants. Twenty-sixth Report of the Joint FAO/WHO Expert Committee on Food Additives, Technical Report series No. 683.
[9] M. Sasaki, K. Sugimura, M. Yoshida, S. Abe, Cytogenetic effects of 60 chemicals on cultured human and Chinese hamster cells, Senshokutai 20 (1980) 574–584.
[10] R.B. Haveland-Smith, Evaluation of the genotoxicity of some natural food colours using bacterial assays, Mutat Res 91 (1981) 285–290.
[11] M. Ishidate, K. Sofuni, K. Yoshikawa, M. Hayashi, M. Nohmi, A. Matsuoka, Primary mutagenicity screening of food additives currently used in Japan, Food Chem Toxicol 22 (1984) 623–636.

[12] H. Fujita, M. Nakano, M. Sasaki, Mutagenicity test of food additives with Salmonella typhimurium TA97 and TA102, Kenkyu Nenpo-Tokyo-Toritsu Elsei Kenkynsho 39 (1988) 343–350.
[13] K. Kovary, T.S. Louvain, M.C. Costa e Silva, F. Albano, B.B.M. Pires, G.A.T. Laranja, C.L.S. Lage, I. Felzenszwalb, Biochemical behaviour of norbixin during in vitro DNA damage induced by reactive oxygen species, Brit J Nutr 85 (2001) 431–440.
[14] P. Palozza, Prooxidant actions of carotenoids in biologic systems, Nutr Rev 56 (1998) 257–265.
[15] P.C.F. Mathias, E.M. Salvato, R. Curi, W.J. Malaisse, A.R. Carpinelli, Effects of epinephrine on ^{86}Rb efflux, ^{45}Ca outflow and insulin release from pancreatic islets perfused in the presence of propanolol, Horm Metab Res 25 (1993) 138–141.
[16] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp Cell Res 175 (1988) 184–191.
[17] E.E.C. (1980). Report of Scientific Committee for Food. Commission of the European Communities, Luxemburg.
[18] R.S. Parker, Carotenoids in human blood and tissues, J Nutr 119 (1989) 101–104.
[19] L.W. Levy, E. Regalado, S. Navarrete, R.H. Watkins, Bixin and norbixin in human plasma: determination and study of the absorption of a single dose of Annatto Food Color, Analyst 12 (1997) 977–980.
[20] C. Jewell, N.M. O'Brien, Effect of dietary supplementation with carotenoids on xenobiotic metabolizing enzymes in the liver, lung, kidney and small intestine of the rat, Brit J Nutr 81 (1999) 235–242.
[21] R.B. Drotman, G.T. Lawhorn, Serum enzymes as indicators of chemical induced liver damage, Drug Chem Toxicol 1 (1978) 163–171.
[22] R.R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J.-C. Ryu, Y.F. Sasaki, Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing, Environ Mol Mut 35 (2000) 206–221.
[23] E.Y.St.A. Morrison, M.E. West, The effect of Bixa orellana (Annatto) on blood sugar levels in the anaesthetized dog, W I Med J, 34 (1985) 38–42.
[24] E.Y.St.A. Morrison, S. Smith-Richardson, M.E. West, S.E.H. Brooks, K. Pascoe, F.R.C.S. Fletcher, Toxicity of the hyperglycaemic-inducing extract of the Annatto (Bixa orellana) in the dog, W I Med J 36 (1987) 99–103.
[25] E.Y.St.A. Morrison, H. Thompson, K. Pascoe, M. West, C. Fletcher, Extraction of a hyperglycaemic principle from the annatto (Bixa orellana), a medicinal plant in the West Indies, Trop Geogr Med 43 (1991) 184–188.
[26] F.G. Plumley, G.W. Smith, Reconstitution of chlorophyll a/b light-harvesting complexes—xanthophylls-dependent assembly and energy-transfer, Proc Natl Acad Sci USA 84 (1987) 146–150.
[27] W. Kuhlbrandt, D.N. Wang, Y. Fujiyoshi, Atomic model of plant light-harvesting complex by electron crystallography, Nature 367 (1994) 614–621.
[28] Y. Cohen, S. Yalovsky, R. Nechushtai, Integration and assembly of photosynthetic protein complexes in chloroplast thylakoid membranes, Biochim Biophys Acta 1241 (1995) 1–30.
[29] G. McDermott, S.M. Prince, A.A. Freer, A.M. Hawthornth-Walte-Lawiess, M.Z. Paplz, R.J. Cogdell, N.A. Isaacs, Crystal-structure of an integral membrane light-harvesting complex from photosynthetic bacteria, Nature 374 (1995) 517–521.
[30] T. Lazrak, A. Milon, G. Wolff, A.M. Albrecht, M. Miehé, G. Ourisson, Y. Nakatam, Comparison of the effects of inserted C-40-terminally and C-50-terminally dihydroxylated carotenoids on the mechanical-properties of various phospholipid-vesicles, Biochim Biophys Acta 903 (1987) 132–141.
[31] A. Wisniewska, W.K. Subczynski, Effects of polar carotenoids on the shape of the hydrophobic barrier of phopholipid bilayers, Biochim Biophys Acta 1368 (1998) 235–246.
[32] C. Socaciu, R. Jessel, H.A. Diehl, Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy, Chem Phys Lipids 106 (2000) 79–88.
[33] W.K. Subczynski, A. Wisniewska, Physical properties of lipid bilayer membranes: relevance to membrane biological functions, Acta Biochim Pol 47 (2000) 613–625.
[34] R.A. Bone, J.T. Landrum, Z. Dixon, Y. Chen, C.M. Llerena, Lutein and zeaxanthin in the eyes, serum and diet of human subjects, Exp Eye Res 71 (2000) 239–245.
[35] F.J. Wolf, Effect of overloading pathways on toxicity, J Environ Pathol Toxicol 3 (1980) 113–134.
[36] P.R. Mayer, Absorption, metabolism, and other factors that influence drug exposure in toxicology studies. Toxicol Pathol 23 (1995) 165–169.
[37] M.S. Christian, Introduction/overview: gender-based differences in pharmacologic and toxicologic responses. Int J Toxicol 20 (2001) 145–148.
[38] R. Czerniak, Gender-based differences in pharmacokinetics in laboratory animal models. Int J Toxicol 20 (2001) 161–163.