ANTIOXIDANT AND EFFECTS OF PROCESSING USING BIXIN POTASSIUM SALT AS A NITRITE REPLACEMENT IN RESTRUCTURED MEAT PRODUCTS

AVALIAÇÃO DO EFEITO ANTIOXIDANTE DO PROCESSAMENTO DE PRODUTOS CÁRNEOS REESTRUTURADOS USANDO SAL POTÁSSICO DE BIXINA COMO SUBSTITUTO DE NITRITO DE SÓDIO

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ABSTRACT
This study aimed to evaluate bixin potassium salt as a replacement for sodium nitrite (NaNO2) in restructured meat products. Bixin was obtained from the chloroform extract of annatto seeds using a Soxhlet apparatus. Restructured meat samples were prepared using raw retail cuts of beef (vastus lateralis), stored at −16º C, and evaluated during storage at 60 days. The efficacy of bixin potassium salt as a NaNO2 replacement in restructured meat was evaluated by measuring residual concentrations of TBARS, color (L*, a*, and b* components), a sensory preference test, and microbiological parameters. Bixin potassium salt proved to be a viable alternative to nitrites for preserving the color and inhibiting the oxidative degradation of restructured meat. The outcomes showed that the effect of bixin potassium salt on microbiological stabilization was quite variable, depending on the bacterial species considered.
Key words: meat, nitrite reduction, carotenoids, annatto, bixin, shelf-life.

RESUMO:
Este estudo teve como objetivo avaliar o sal potássico da bixina como substituto do nitrito de sódio ($NaNO_2$) em produtos cárneos reestruturados. A bixina foi obtida a partir do extrato clorofórmico de sementes de urucum utilizando um aparato de Soxhlet. Amostras de carnes reestruturada foram preparadas com cortes crus de carne bovina (*vastus lateralis*), armazenadas a -16º C, e avaliadas durante o armazenamento durante 60 dias. A eficácia do sal potássico da bixina na substituição de $NaNO_2$ na carne reestruturada foi avaliada através da medição das concentrações residuais de TBARS, componentes de cor (L *, a * e b *), um teste de preferência sensorial e parâmetros microbiológicos. O sal potássico da bixina provou ser uma alternativa viável ao nitrito para preservar a cor e inibir a degradação

oxidativa da carne reestruturada. Os resultados mostraram que o efeito do sal potássico da bixina na estabilização microbiológica foi bastante variável, dependendo da espécie bacteriana considerada.
Palavras-chave: carne, redução de nitrito, carotenoides, urucum, bixina, prazo de validade.

1. INTRODUCTION

Bixin is a carotenoid extracted from the seeds of B. orellana and used as an FDA-approved food colorant and additive, as well as a cosmetic and textile colorant (JONDIKO & PATTENDEN, 1989; MERCADANTE, RODRIGUEZ-AMAYA, PFANDER, & BRITTON, 1996; ULBRICHT et al. 2012).Importantly, its acceptable daily intake (ADI) over a lifetime without an appreciable health risk surpasses that of any other carotenoid approved as a food additive [ADI (bixin): 12 mg/kg body weight/day] WHO (2011).

Several studies have shown that carotenoids can act as natural chain-breaking antioxidants by scavenging and deactivating free radicals both in vitro and in vivo1,2. Annatto dye contains a series of carotenoid-type pigments extracted from the seeds of the bush Bixa orellana L., which imparts a yellow-to-red color to food3. Annatto (characterized by different traditional names, such as “onoto” in Venezuela or “achiote” in Mexico and “colorau” in Brazil, depending on its geographic origins) is the common name of a natural condiment/colorant used worldwide, inserted in many culinary traditions, used as a food additive (classified as E160b in Europe), and working as a yellow-orange colorant (permitted in butter and margarine for example) (BEMRAH et al. 2012).

Bixin is safe and healthy and has been traditionally used to treat chest pain and infectious and inflammatory diseases of the skin, prostate, and gastrointestinal tract. In recently published studies, bixin has demonstrated antigenotoxic and antioxidant cytoprotect activities and skin photoprotection, and systemic availability of oral bixin and its metabolite demethylated, norbixin, has been documented in rodent and healthy human study subjects (TAO et al., 2015, 2016; ULBRICHT et al., 2012).

Preservatives and dyes are increasingly used by the food industry, and there is a growing tendency to shift from synthetic to natural agents endowed with the same properties. The identification and characterization of safe preservatives characterized by antimicrobial activity (better if simultaneously working as antioxidant) isolated from natural sources is a significant challenge for food technology today. One significant and challenging example is the partial replacement of nitrite by natural antioxidants to preserve meat color (ALAHAKOON et al., 2015; BARBIERI et al., 2013), hypothetically preserving the meat from microbiological risk (particularly concerning Clostridium botulinum). In this way, the

carcinogenic, mutagenic, and genotoxic activities of nitrosamines that can be formed from nitrites are avoided. This alternative has been investigated by different researchers (ALAHAKOON et al., 2015; BARBIERI et al., 2013; BISWAS et al., 2012; CASTRO et al., 2011; WALTERS, 1992; ZARRINGHALAMI et al. 2009).

The principal aim of this study was the evaluation of bixin as a total or partial replacement for sodium nitrite (NaNO2) in restructured meat products in order to check color stability, oxidative status preservation, and microbiological protection, potentially assessing a new protocol to make “safe” processed meat.

2. MATERIAL AND METHODS

2.1. Isolation of bixin

A bixin standard was obtaining according to the method of GOLIN, ROCHA GARCIA, BARREIRA, BEDNARCZUK, STRAPASSON, ZUCHETTO & MIGUEL, 2013.

2.2. Salification of bixin

The conversion of bixin into a more soluble salt was done by treating a sample of bixin (2 g) in 10% KOH/MeOH (p/v). After stirring for 5h at room temperature, the precipitate was filtered over a G3 sintered glass funnel. The precipitate was dried in an oven at 50°C for 24 h, to yield 80%.

2.3. Characterization of bixin and bixin potassium salt

IR spectra were registered on Bomem-Hartmann and Braun equipment MB Biored FTS 3500 GX, while reading the transmittance between 400 and 4000 cm^{-1}.

All NMR experiments were performed on a Bruker AVANCE III HD 600 NMR spectrometer operating at 14.1 T, observing ^{1}H and ^{13}C at 600.13 and 150.90MHz respectively. The spectrometer was equipped with a 5mm quadrinuclear inverse detection probe with a z-gradient. The ^{1}H and ^{13}C $\{^{1}H\}$ spectra were acquired on a spectral width of ~11 ppm and ~240 ppm. One-bond (HSQC) and long-range (HMBC) ^{1}H-^{13}C NMR correlation experiments were optimized for average coupling constants $^{1}J_{(H,C)}$ and $^{LR}J_{(H,C)}$ of 140 and 8 Hz respectively. All ^{1}H and ^{13}C NMR chemical shifts were observed in ppm related to the TMS signal at 0.00ppm as internal reference. DMSO-d6 was used as solvent.

The chromatographic analysis was done in a high efficiency liquid chromatographer (Pro Star Gradient VARIAN, model 410, 50492 series) with a photodiode array detector (model 335) using a reverse phase C18 column (VARIAN, 5µm, 150 x 4.6 mm), working at 29°C in isocratic mode. The mobile phase was 85% acetonitrile (J.T. Baker USA) – 15% acidic water (0.05M H_2SO_4 and 1% H_3PO_4). The flow was 1 mL/minute and the wavelength 470nm with 8 minutes running time. All samples were dissolved in methanol (J.T. Baker Mexico) before the analysis.

The analytical curve was prepared between 2.45 and 78.4µm/mL (n=7) by means of the adapted method by Scotter, Thorpe, Reynolds, Wilson & Strutt (1994) and Scotter, Castle & Appleton (2001), using bixin and bixin potassium salt in methanol.

2.4 Sample preparation

Approximately 15 kg of raw bovine beef (*Vastus lateralis*) samples bought in a Curitiba, was used to produce restructured meat products. The meat was first milled in a 3.5 mm diameter disc in an industrial mill. The meat mass was homogenized using different combinations of bixin and nitrite.

The control (CTRL) was developed without any nitrite and bixin potassium salt combination. A batch of samples (NIT) was developed just with sodium nitrite (150 ppm). An experimental mixture (BIXNIT) was then developed by mixing nitrite (75 ppm) and bixin potassium salt (250 ppm). Finally, exploiting a third treatment (BIX), nitrite was totally replaced by bixin potassium salt (500 ppm). The additive combinations were diluted in water, and the volume ratio was 10% of the meat mass (p/v). Each group was composed by 3.5kg cuts of beef (*Vastus lateralis*). Subsequently, the beef samples were homogenized with their respective additive combinations for five minutes, then weighed (100g±0.1) and molded into hamburger patties.

The preparation time average was about two hours. Samples were stored at −16°C (±1°C) up to the end of the shelf-life analysis.

2.5. Protein content

Protein determination was done by a five-time repetition according to the Kjeldahl method by adopting the 6.5 factor for nitrogen conversion, according to AOAC (1997).

2.6. Lipid and moisture content

Total lipids were determined according to the Soxhlet method described by the AOAC (1997).

2.7. Fixed mineral residue determination

The determination of the fixed mineral residue was done by a five-time repetition by muffle furnace (ashes) according to the method described by AOAC (1997).

2.8. pH determination

pH was determined by a five-time repetition and measured by a potentiometer (Hanna) – HI 8313, after calibration to 4.0 and 7.0 pH, according to Chemists & Horwitz (1997).

2.9. Color measurement

The L* (lightness), a* (redness), and b* (yellowness) values were measured by reflectometry in off-specular mode with the illuminant D65 and an observation angle of 10° in a Hunter colorimeter (Hunter Associates Laboratory, Inc., Virginia. USA). The reported values are means of five measurements performed at different points (5cm² overtures) on the surface of different raw, frozen, and fresh samples WANOUS et al. (1989).

2.10. Analysis of thiobarbituric acid reactive substances (TBARS)

The determination of thiobarbituric acid reactive substances was performed according to Tarladgis Pearson & Jun Dugan (1964) and Madsen, Sorensen, Skibsted& Bertelsen(1998). The standard curve was established using 1, 1, 3, 3-tetraethoxypropane (TEP, Sigma-Aldrich, USA) in the concentration range from 0.10 to 7.0 M. The results were expressed in mg (TBARS) per kg of the sample. The analyses were carried out in triplicate using three different samples: raw, frozen and fresh.

2.11. Evaluation of the nitrite residual concentration

The restructured products were homogenized and 10 grams (± 0.001) of each sample

were collected; 5.0 mL of sodium tetraborate and 50.0 mL of water at 80 °C were added to them. After 15 minutes at 80 °C in double boiler in agitation (Solab, model SL-155/22-8), the samples were transferred to a volumetric flask and 5.0 mL of potassium ferrocyanide and 5.0 mL of zinc acetate were added to them. They were subjected to 2 minutes agitation between each reagent addition. The volume was completed up to 250 mL with distilled water, leaving quiescent the samples for 30 minutes. After filtration, an aliquot of 10 mL was collected; α-naphtol (Vetec) and sulfanilamide in acid medium were added, in order to develop color after filtration. Spetrophotometer was then used to measure absorbance at 540 nm. The amount of nitrite were then assessed by means of the previously established standard curve by Instituto Adolfo Lutz (2005).

2.12. Sensory analysis

The sensorial evaluation was made using a preference test. Tasters scored their preferences from 1 (disliked extremely) to 4 (liked extremely) on a consumer preference questionnaire for raw, frozen and fresh samples formulated according to four different treatments (CTRL; BIX; BIXNIT; NIT). The total score was finally used to indicate the samples most appreciated by the tasters (INSTITUTO ADOLFO LUTZ ,2005).

2.13. Microbiological analysis

The microbiological analysis was done in the Istituto Zooprofilattico Sperimentale Piemonte, Liguria and Valle d'Aosta di Torino (Italy). In order to verify the inhibition of pathogens by bixin and sodium nitrite, a cocktail made up of three strains of *Listeria monocytogenes* (ATCC® 7644; IZSPLV 85691/3-2014; IZSPLV 87357-2014), *Escherichia coli* O157 (NCTC® 12900; IZSPLV 4156-2016; IZSPLV 1963-2016), *Clostridium perfringens* (ATCC® 13124; IZSPLV 59757-2014; IZSPLV 50756-2014) and *Salmonellaenterica* (ATCC® 13076, *serovar Enteritidis*; IZSPLV 6174-2015, *serovar* 4[5],12:i:-; IZSPLV 10971-2015, *serovar Typhimurium*) was used. All strains were available as a part of the frozen stock collection at -20 °C at Cryobank™ (Copan Diagnostics, Murrieta, CA). For each pathogen, two independent trials were carried out. In each trial, the strain was resuscitated twice in Brain Heart Infusion (BHI) broth (Microbiol, Cagliari, Italy) and incubated at 37°C for 24 h under aerobic (*L. monocytogenes, E. coli* O157 and *S. enterica*) and anaerobic conditions (*C. perfringens*). For each pathogen, a cocktail of three strains was obtained by

mixing 1mL of each. Decimal dilution was performed to achieve a final concentration of approximately 1.5-2.0 log cells/mL in the specific broth. Four broths with different time.

The broths were incubated as described above for BHI. After that, *L. monocytogenes*, *E. coli* O157, *S. enterica*, and *C. perfringens* were counted using the pour plate technique respectively on Agar Listeria acc. to Ottaviani and Agosti (Biolife, Milan, Italy), CHROMagar^{TM} O157 (CHROMagar, Paris, France), Xylose Lysine Deoxycholate Agar (Biolife, Milan, Italy) and Tryptose Sulphite Cycloserine (Biolife, Milan, Italy).

2.14. Statistical analysis

The results of chemical composition, color, TBARS and the sensorial analysis of the treated material and the raw, frozen and fresh samples were subjected to variance analysis and to the comparison of averages using the Turkey test at 5% significance level and applying ANOVA. The Kruskal-Wallis non-parametric test, which is similar to ANOVA, was also used in cases in which one of the assumptions was not found (Gaussianity and homoscedasticity). The basic assumptions for the t-test were checked by applying the correlation test (Handbook of the MINITAB software) to Gaussianity in each level of the pair and the F and L even tests to homoscedasticity in the MINITAB software (STATSOFT, 1995).

2.15 Technical aspects

Volunteers did the sensorial analyses. Therefore, the current research was submitted to and approved by the Ethics Committee in Health Science of Federal University of Paraná – CAAE: 22206713.9.0000.0102.

3. Results and discussion

In order to establish the capacity of bixin potassium salt to act as microbial stabilizer in processed meat, thus potentially reducing the dose of nitrites added to the food matrix, this work started with the preparation of natural bixin from annatto seeds. The red-purple powder of bixin (yield: 5.1%) was obtained according to the method of Golin, S., Rocha Garcia, C., Barreira, S., Bednarczuk, V., Strapasson, G., Zuchetto, M., & Miguel, O, (2013). The polarity gradient was performed in order to certify that bixin would not be present in other solvents such as ethyl acetate and ethanol. The material was oven-dried and stored

at −16ºC. The bixin so prepared has been characterized: first, the measured melting point of bixin (195°C) was similar to the one previously reported in the literature(Golin, S., Rocha Garcia, C., Barreira, S., Bednarczuk, V., Strapasson, G., Zuchetto, M., & Miguel, O, 2013; Jondiko & Pattenden, G, 1989; Mercadante Steck, A., Rodriguez-Amaya, D., Pfander, H., & Britton, G., 1996; Rodrigues et al., 2014). The identification was further supported by Nuclear Magnetic Resonance spectroscopy (^{1}H and ^{13}C NMR) as well as by UV and IR (JONDIKO & PATTENDEN, 1989; REHBEIN et al. 2007).

3.1 Bixin potassium salt synthesis and its identification

Bixin potassium salt, a more polar version of the natural product isolated from annatto, was prepared to increase the polarity of bixin and to intensify its color (to improve the color capacity). Remarkably, norbixin (the mono-esterified form of bixin) has a typical orange color, a fact that could negatively influence product acceptance by consumersNoppe et al. (2009 e Scotter et al. (2001). The absorptions at the maximum lambda in the UV region for both bixin and its salt were similar Jondiko & Pattenden, G (1989 e Noppe et al. (2009 e Scotter et al. (2001), and their purity was estimated >99%. UV$_{\lambda}$, nm: 489, 462, 432,7. IR υ(KBr) cm^{-1}1716.64, 1660, 1614, 1385, 1300 and 900. ^{1}H NMR (600MHz. DMSO-d6), δ 7.89 (1H*, d, J*=15.5Hz, H-7), δ7.26 (1H*, d, J*=15.5Hz, H-7'), 6.45-6.87(10H, *m,* 10x :CH), 5.83 (1H*, d, J*=15.5Hz, H-8), 5.94(1H*, d, J*=15.5Hz, H-8'), 3.70 (3H, *s,* OMe), 1.92-1.99 (12H, *m*, 4x: CMe).

The infrared spectroscopy of the characteristic strong absorption due to the C=O stretching frequency between 1740 and 1700 cm^{-1}and the complex bands in the 1300–1050 cm^{-1} region due to C-O single bond characteristic of ester and carboxylic acids has been used (JONDIKO & PATTENDEN, 1989).

3.2. Physico-chemical characterization of restructured raw beef

The analysis of the proximate composition of raw beef (*vastus lateralis*) showed 21.02% and 1.15% for protein and lipids respectively (fresh weight). The measured mineral content (evaluated as ashes content, fresh weight) was 1.09%; all the data recovered were in accordance with NEPA, 2004. Restructured meat showed a loss of proteins (-3.78%) and a correlated increase in lipids and ashes.

The pH of the meat samples used in this model study was 5.66; the pH measured in restructured meat was 5.79. As is well known, the post-mortem pH of meat is set by the

amount of lactic acid produced from glycogen during anaerobic glycolysis, and it may be stopped if the glycogen is consumed by fatigue, inanition, or by the animal's fear before slaughter. The final pH of the meat affects both its texture and its microbial quality; in fact, pH is a key factor in modulating the microbial growth in the meat matrix (YANG et al. (2014).

Most bacteria grow in pH 7.0. Whenever the pH is under 4.0 or above 9.0, bacteria face resistance to proliferation, even if more parameters affect microbial growth besides acidity or alkalinity. Fresh bovine meat generally maintain pH between 5.3 and 6.5, depending on the care taken of the animal before slaughter (rest, fast, stress) and on the following biochemical changes (ALAHAKOON et al. 2015; SCOTT & TAYLOR, 1981).

3.3. Nitrite residual concentration in restructured meat products

The model food 150 mg/Kg of nitrite ($NaNO_2$) were added in (restructured meat) as reference treated meat; 75 mg/Kg were added to the sample were bixin were used (250 mg/Kg). Moreover, a sample where any addiction has been made, and one where only bixin was added (500 mg/Kg) completed the series of model foods here considered. The concentrations of the nitrite salt, as well as the concentration of the bixin potassium salt were arbitrarily decided considering technological practices and previous scientific works. The key target of this work, as previously introduced, was to evaluate the chance to reduce nitrite salt using "natural ingredients" bixin potassium salt considering both processing and storage/ripening. Nitrite residual levels were reduced to approximately 2/3 of the original concentration by the end of the process. After 7 days at -16 °C (frozen storage), the nitrite content showed a 50% reduction($p<0.05$).

According to analyses done between the 30^{th} and 60^{th} storage day, only traces of nitrite were found (Table 1). The demanding nitrite concentration for technical use in meat products varies between 30 and 50 ppm, in order to properly stabilize color (a secondary "technical effect" of this additive). Furthermore, a range between 80 and 150 ppm is generally functional to obtain a good preservation effect, and, finally, a range between 20 and 50 ppm is technically useful to obtain the correct antioxidant effect (REDDY LANCASTER & CORNFORTH, 1983). Nitrite consumption can be due to the action of reducing substances endogenous to the meat, e.g. sulphur- containing amino acids (cysteine), or added ones such as ascorbate. In addition, a nitrite dismutation reaction also results in nitrate formation. The consumption of NO occurs by a reaction with both the denatured pigment of meat and with some substrates present in the mixture, such as the biochemical cellular systems of microorganisms, which pre- vents their growth and

preserves the meat product from a microbiological point of view (BARBIERI et al., 2013; REDDY LANCASTER & CORNFORTH, 1983).

Table 1 - Residual nitrite in meat restructured samples made with bixin potassium salt

Sample		Day			
		0	7	30	60
	Original concentration		Residual nitrite/ppm		
MP	-	-	-	-	-
CTRL	-	-	-	-	-
BIX	-	-	-	-	-
BIXNIT	75 ppm	43,25[a]	30,62[b]	ND	ND
NIT	150 ppm	101,89[a]	63,29[b]	ND	ND

a, bMeans in triplicate followed by same letter in line do not differ significantly (p> 0.05) Time "0" = fresh sample just prepared. MP= feedstock; CTRL=control; BIX =(500 ppm bixin potassium salt); BIXNIT= (250 ppm bixin potassium salt 75 ppm NaNO2); NIT=(150 ppm NaNO2); ND = not detectable.

3.4. TBA analysis

The oxidative state of processed meat is a key point regarding sensorial profiling and affects both the color and the flavor of meat products. The level of meat oxidation is generally reduced by the use of additives (antioxidants). The secondary aim of this work, besides microbiological monitoring, was to assess the oxidative degree of the processed samples. Malondialdehyde recovery obtained by means of the methodology used in the distillation process was 96%, and the correction factor used was 4.67. The lowest oxidation levels were found in samples containing sodium nitrite, as expected. During the whole storage time, the processed meat treated with curing-salt presented less oxidation—from 2.5 (at the end of the processing) to 4.3 (60^{th} storage day)—when compared to the control (Fig. 1).

Except for the beginning of the process (Time 0), the bixin potassium salt used at a concentration of 250 ppm potassium bixin potassium salt: 75 ppm $NaNO_2$ showed an antioxidant effect lower than that provided by nitrite, as expected. However, during the assessed period it provided a significant antioxidant action ($p<0.05$) when compared to the control, without any additives. At the end of the storage time, when analyzing rancidity in the control, nitrite (150 ppm) reduced the oxidation in the sample. When nitrite was partially replaced (75 ppm) by bixin potassium salt (250 ppm) such protection was reduced to approximately 67% ($p<0.05$). Moreover, the total nitrite replacement (0 ppm) by bixin

potassium salt (500 ppm) allowed approximately a 30% protection from oxidation ($p<0.05$) when compared with the sample without any protection. Figure 1 shows the TBARS values measured on the samples considered in this study.

Annatto's anti-oxidation activity, even if not in the form of bixin potassium salt, was found and demonstrated in other meat products such as sausages (MERCADANTE et al.; 2010; ZARRINGHALAMi et al. 2009) and hamburger patties. As reported by Castro et al (2011), a concentration of (0.4g/100g) of annatto (containing 173 ± 24mg/100g of bixin) extract was used, allowing a significant protection of lipid oxidation, color stability, and degradation of bixin, and vitamin E was investigated in raw and grilled patties during storage at −18°C for 120 days.

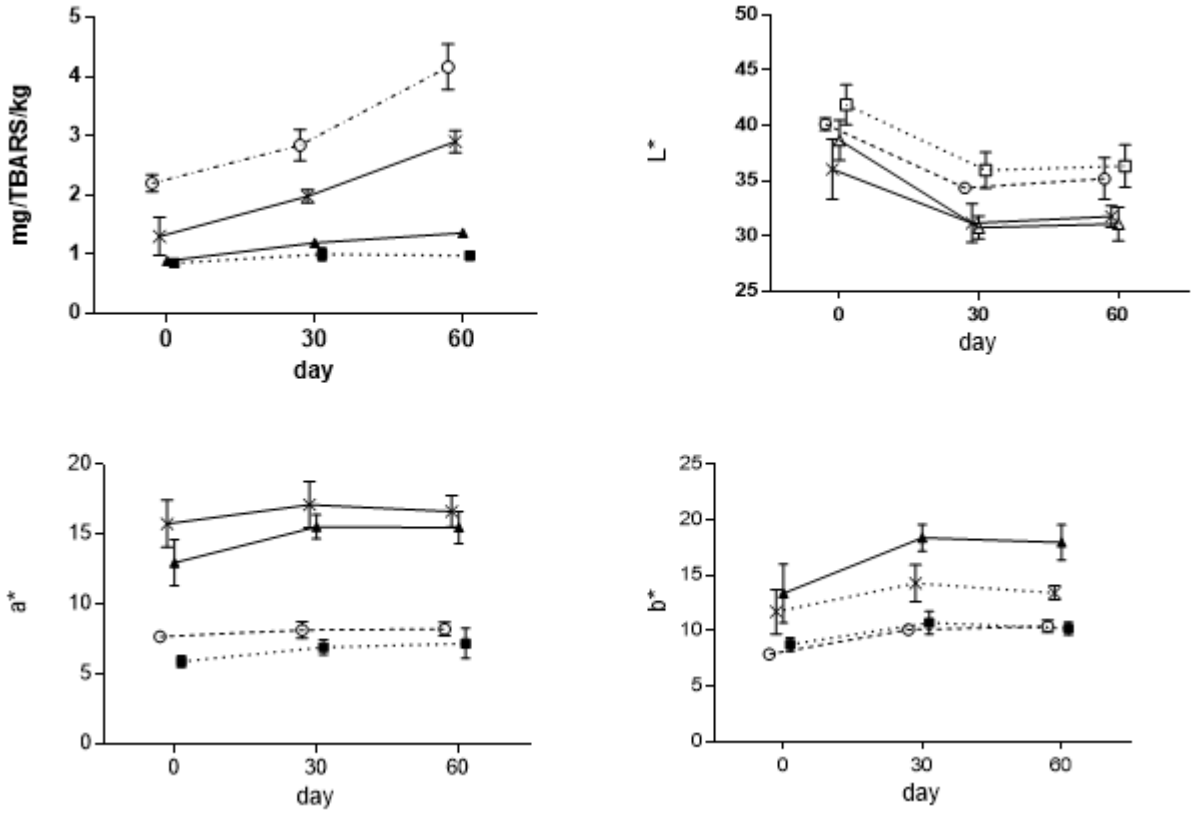

Fig.1 Development of lipid oxidation in raw restructured beef products measured as TBARS, (n=6) L* (brightness), Colour stability (a*) and (b*) (n=9) during storage at -16°C for 60 days. The bars are the standard deviations. CONTROL (o), BIX (x), BIXNIT (▲), NIT (■).

3.5. Color evaluation

3.5.1 Effect of nitrite replacement by bixin potassium salton the color of restructured products

Lightness evaluation (L*) in unripe restructured meat products often showed that bixin potassium salt reduces L* in the samples (Fig. 1). Total (BIX) or partial nitrite (BIX/NIT) replacement provided opaque products when comparing them to the control or to formulations containing just nitrite (NIT), thus decreasing L* measured values. Storage time also leads to lightness reduction in the samples. Almost all the treatments showed significant reduction in L* values during the first 30 days; following storage, L* showed stabilization. A significant reduction of the lightness parameter during storage indicates opacity in the samples. Such a condition is strictly related to the superficial oxidation process in all the samples. These significant differences were observed after the first 30 storage days only in samples formulated without bixin (CTRL and NIT) (Fig. 1).

Samples containing just nitrite (NIT) presented the lowest a* values during storage. The red color in this case appeared to be lower than that of the control (Fig. 1). Sodium nitrite is not the appropriate dye to be used in unripe meat products. The NO bound provided by nitrite to myoglobin gives the characteristic red color to meat products. However, this bound is preceded by the oxidation of iron found in the porphyrin ring. Such change leads to opacity in the meat (RAMÍREZ ESTÉVEZ, MORCUENDE & CAVA, 2004). Treatments such as salting, or cooking favor the interaction between myoglobin, nitrite, and the red color. However, the brown color remains in the unripe products. The combined use of nitrite and antioxidants such as sodium erythorbate also appeared to be effective in increasing the a* parameter in meat products(BARBIERI et al. 2013; RUSSELL et al. 2004).

The replacement of sodium nitrite by bixin potassium salt favored the red color in the raw meat samples. Samples containing bixin presented a higher a* parameter depending on the higher concentration of this salt (BIX>BIXNIT). The color was not impaired during the first 60 evaluated days. Compared to the control, samples containing bixin potassium salt presented almost double the a* parameter during their shelf-life. A value of 8.24 a* was found in CTRL after 60^{t} days of storage, whereas BIX and BIXNIT reached the respective averages 16.66 and 15.51 in the same samples. These values demonstrate the effectiveness of bixin potassium salt and its stability when combined with the meat dye.

Bixin potassium salt, besides providing the red color and improving the acceptability

of the product under the consumer science umbrella, also appeared to provide a yellow color tone in raw samples (Fig. 1). Samples containing bixin potassium salt presented higher b* values throughout the storage time when compared to NIT or CTRL treatments. The stability of the color provided by bixin potassium salt during the 60 storage days was also noticed.

One of the great concerns regarding natural additives was instability during storage. Color stability evaluation in sausages showed in previous works that after 45 days storage at 4 °C, natural dyes such as zeaxanthin, norbixin, carotene, and lycopene presented color loss due to susceptibility to the oxygen oxidative action. However, throughout the sensorial tests, such color change was identified and voted as “quite pleasant” by chefs and cooks even after 45 days of storage at 4°C (MERCADANTE et al. 2010).

Moreover, the vitamin E antioxidant effect and the use of packages with different modified atmosphere conditions were compared in order to check color stability in meat products frozen for 18 days. The reduction in oxygen concentration and the presence of tocopherols were effective in preserving the color of meat products throughout storage. (CASTRO et al. 2011). Sant’Anna et al. (2013), in a review on natural dyes in food, suggest that color monitoring could be a useful approach to have real time control over the quality of technological functions of natural dyes. All these findings confirm our observation: the use of a natural color, like bixin, despite the sensitive effect of oxygen on the product, can be considered a functional alternative to preserve color in processed meat, at least during the period considered in this research (ALAHAKOON et al. 2015; NASSU et al. 2012).

3.6. Sensorial analysis

3.6.1 Preference tests

The visual sensorial analysis of the raw, frozen and fresh products showed that samples containing just sodium nitrite (NIT) throughout the storage period were less accepted ($p<0.05$) when compared to the control or to samples treated with bixin potassium salt. NIT (47) was overcome by BIX (75) or BIXNIT (91) as well as by CTRL (97) in raw presented samples by the end of the process. Such behavior remained consistent, and again NIT (45) was overtaken ($p<0.05$) by BIX (83), BIXNIT (80) or CTRL (82) by the end of evaluated period (Fig. 2).

Unripe meat samples containing nitrite presented a brownish shade due to iron oxidation to iron status (Fe^{3+}). The meat color becomes unacceptable to most of the

consumers whenever approximately 50% of the myoglobin is found in its metmyoglobin form (ALAHAKOON et al. 2015; YOUSSEF, GARCIA & SHIMOKOMAKI, 2003). The raw samples formulated using just nitrite (NIT) have triggered lower preference values and also a lower ($p<0.05$) intensity of the red color (a* parameter) when compared to treatments using bixin potassium salt (Fig. 2).

There are air incorporations during most of the processing of ripe products and during meat mass milling. It provides oxygen to the tissues and, thus, the oxymyoglobin will be predominant by the time the ingredients used to ripen the mass are added to it. Initially, the oxymyoglobin and the myoglobin are oxidized to metmyoglobin by nitrite action. Subsequently, the metmyoglobin reacts with nitrite oxide, and they form the nitrosyl metmyoglobin complex. The reduction of nitrosyl metmyoglobin, due to the action of reducing enzymes, reduces agents such as potassium ascorbate or sulfhydryl groups released during the heat treatment, or the straight reaction between myoglobin and nitrite oxide will originate nitrosyl myoglobin (ALAHAKOON et al. 2015; BARBIERI et al. 2013; FOX & ACKERMAN, 1968; KOIZUMI & BROWN, 1971; YOUSSEF, GARCIA & SHIMOKOMAKI, 2003).

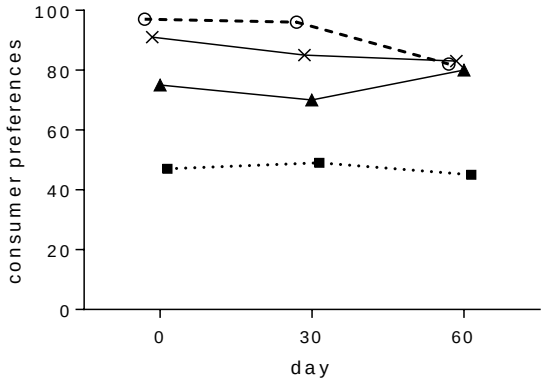

Fig.2. Consumer preferences of restructured meat products during storage at -16°C for 60 days. Legend: CONTROL (o), BIX (x), BIXNIT (▲), NIT (■).
The bars are the standard deviations (n=30).

3.7. Microbiological analysis

As described in the Material Methods session, series of microbiological analysis was performed in order to evaluate more deeply the effect of bixin as a co-additive in antimicrobial activity. Table 2 reports the results obtained using specific strains belonging to

L. monocytogenes, *E. coli* O157, *C. perfrigens*, and *S. enterica*.

As reported in Table 3*, E. coli* O157, *C. perfringens*, and *S. enterica* were not inhibited by bixin and $NaNO_2$ when used at these concentrations. Of the organisms tested, only *L. monocytogenes* was inhibited by concentrations of $NaNO_2$ (150 ppm) and the combination of $NaNO_2$ (75 ppm) and bixin (250 ppm). On the other hand, no effect was observed in the BIX broth. This study confirmed that ingoing nitrite concentration influences *L. monocytogenes* growth as observed previously (KING et al. 2016), but single bixin potassium salt is not able to inhibit pathogen growth. The results show how the use of bixin could allow a reduction in the use of nitrite. As described in previous works and in different food matrices, the effect of natural antimicrobial compounds can be considered specific for some microbial species, as well as for a specific range of concentrations. These results indicate that the deepening of this aspect due to reducing additives in meat products, such as nitrite, is desirable.

Table 2. Microbiological results (Mean ± Standard Deviation)

Medium	*L. monocytogenes* (cfu/ml)	*E. coli* O157 (cfu/ml)	*C. perfringens* (cfu/ml)	*S. enterica* (cfu/ml)
Initial concentration	2.2 ± 0.1	1.7 ± 0.1	1.5 ± 0.2	2.0 ± 0.1
Negative control	9.2 ± 0.2	8.9 ± 0.1	8.6 ± 0.2	9.0 ± 0.1
BIX	9.2 ± 0.1	8.9 ± 0.1	8.5 ± 0.1	9.1 ± 0.2
BIXNIT	7.4 ± 0.2	9.0 ± 0.1	8.6 ± 0.1	8.9 ± 0.2
NIT	7.1 ± 0.1	9.1 ± 0.1	8.6 ± 0.2	8.9 ± 0.1

4. CONCLUSION

Bixin potassium salt qualifies as a nitrite alternative to preserve the red color of meat and to avoid the oxidative degradation of raw, frozen, and fresh restructured meat products. Yellow-orange dyes derived from annatto are known as technological tools and their use is regulated by the legislation. However, bixin potassium salt is a novel, purple-red, and more polar analogue that deserves consideration as a superior annatto-based dye for food use.

Even if the antioxidant properties of bixin are lower than those of nitrites, they can nevertheless increase the shelf life of meat products, replacing or reducing the use of nitrites in cured meat products. Despite these results, the effect of bixin potassium salt at the concentrations used in this work, with or without nitrites, can modulate the microbial species

in processed meat, even if a specific action towards pathogens was not shown. More studies are required to optimize the reduction of nitrite content in processed meat, allowing new functional processing methods useful to improve the shelf life of restructured meat products.

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6. CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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