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Digestive Stability and Transport of Norbixin, a 24-Carbon Carotenoid, across Monolayers of Caco-2 Cells

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Abstract

Annatto is a natural pigment widely used in the food industry to add yellow to red colors to dairy and cereal products. Here, the *in vitro* bioaccessibility and potential bioavailability of norbixin, the abundant 24-carbon carotenoid in annatto, were investigated. Norbixin added to milk was highly stable during simulated digestion, and bile salts enhanced partitioning of this carotenoid in the aqueous fraction during the small intestinal phase of digestion. Apical uptake of norbixin by Caco-2 cells was proportional to the concentration in apical medium, but cellular content increased only slightly after 60 min. Transport of norbixin to the basolateral compartment was maximum at 120 min. Both all-*trans* and *cis* isomers of norbixin were present in cells and basolateral medium. The results suggest that ingested norbixin is stable during gastric and small intestinal phases of digestion and that both *cis* and all-*trans* isomers are bioavailable.

Keywords

Annatto; norbixin; digestion; bioavailability; Caco-2 cells

INTRODUCTION

The expanding research on the health-promoting activities and antioxidant capacity of natural pigments, as well as the toxicity of some synthetic dyes, has stimulated consumer interest in products containing natural color additives (1, 2). The annatto pigment has been extensively used as a natural coloring agent and medicinal product in native cultures. Annatto is extracted from the pericarp of the annatto seeds of *Bixa orellana*, a shrub native to tropical Latin America, and is extensively used in the modern food industry to color some dairy and cereal products (3).

Norbixin (NBX) is a 24-carbon carotenoid with carboxylic acids at both termini that is abundant in annatto (Figure 1). This amphipathic molecule contains the multiple conjugated unsaturated double bond system that is characteristic of carotenoids and confers radical scavenging activity (2–6). NBX exists in different isomeric conformations (Figure 1) as a result of the double bonds in the aliphatic chain (7). It has been reported that NBX is an efficient antioxidant against oxidative deterioration of lipids and has greater antioxidant

activity than lutein, β -carotene, and lycopene in oil-in-water emulsion systems (5, 6). The antimutagenic potential of NBX has been related to its capacity to scavenge singlet O_2 (8). The antimicrobial activity of annatto pigment has been attributed to both the 9'-*cis* and the all-*trans* isomers of NBX (9, 10). Whether the above activities occur in vivo remains unknown and are dependent on the absorption and delivery of these isomers to tissues.

Delivery of 40-carbon carotenoids to target tissues requires partitioning into mixed bile salt micelles during digestion, uptake by enterocytes in the small intestine, and transport in chylomicrons into the lymph for delivery to peripheral organs (11). However, information on the absorption of natural carotenoids with shorter aliphatic chains and amphipathic characteristics such as NBX is quite limited. The labor intensity and expense of determining the bioavailability of dietary compounds has provided the impetus for the use of in vitro models that simulate the digestion and intestinal cell uptake of dietary compounds. The transfer of carotenoids in foods and meals during simulated digestion into micelles has been validated as a cost-effective tool for predicting the in vivo bioavailability of carotenoids (12). Furthermore, coupling in vitro digestion with the Caco-2 human intestinal cell model provides the opportunity to examine the uptake and transport of carotenoids from digested foods, formulations, and beverages (13).

Here, we report novel results about the digestive stability and micellarization of NBX during simulated digestion of milk containing annatto, as well as the characteristics of uptake and *trans*-epithelial transport of NBX isomers by monolayers of differentiated Caco-2 human intestinal cells.

MATERIALS AND METHODS

Chemicals

Commercial alkali-extracted NBX (Annatto 15%) was generously provided by Chris Hansen Inc. (Milwaukee, WI). NBX and bixin standards were purchased from Chromadex (Santa Ana, CA). Unless indicated, all other chemicals were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Co. (Norcross, GA). Sodium hydroxide was purchased from Janeille Chemical Co. (Cincinnati, OH). All chemicals and solvents were ACS and high-performance liquid chromatography (HPLC) grade. Milk (skim, 2%, and whole) was purchased at a local grocery in Columbus, OH.

Preparation of NBX-Enriched Milk

Aqueous stock solutions of NBX (90.5% 9'-*cis*-NBX) were prepared in deionized water. The concentration of NBX in the stock solution was determined using the NBX standard (97.1% 9'-*cis*-NBX) as a reference and a HP 8453 UV/vis spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) at 453 nm with 0.1 M NaOH as the solvent and $E_{1\%} = 2850$ as the extinction coefficient (14). The NBX stock solution was mixed with an equal volume of either skim, 2%, or whole milk to achieve the indicated concentrations of the pigment.

In Vitro Digestion

Aliquots of test samples of NBX-enriched milk were subjected to simulated gastric and small intestinal digestion as described previously by Garrett et al. (15). Test milk (4 g) containing 150–180 μ g of NBX was diluted to a final volume of 50 mL during the digestion process. Final concentrations of porcine pepsin, porcine pancreatic extract, bile salt-dependent lipase, and porcine bile extract for digestion were 2.0, 0.4, 0.2, and 2.4 mg/mL, respectively. Bile extract was omitted during small intestinal phase of digestion in one experiment to determine the extent of partitioning of NBX in the aqueous fraction in the absence of mixed bile salt micelle formation. Following digestion, aliquots (10 mL) of

digesta from each reaction tube were centrifuged at 5000g for 45 min at 4 °C to collect and filter (Millex GP, 33 mm diameter, 0.22 μ m pore size, Millipore, Billerica, MA) the aqueous fraction. We recently reported that the quantity of carotenoids partitioning in the aqueous fraction in samples digested at 5000g is not significantly different from that after isolation of the aqueous fraction from digesta at 166000g (16). Aliquots of test milk samples, digesta, and filtered aqueous fractions were flushed with N₂ to minimize oxidation and stored at -20 °C for a maximum of 1 week.

Uptake and Transport of NBX by Caco-2 Cells

Maintenance of Caco-2 cell cultures (HTB-37, ATCC) has been described previously (15, 17). Wells (9.6 cm²) and Transwell inserts (4.9 cm²) were seeded with 5.5×10^4 and 2.6×10^4 cells/cm², respectively. The initial uptake experiments were performed using differentiated monolayers adhered to the plastic surface of six-well dishes at 11–14 days postconfluency (15). Test medium was prepared by 1:4 dilution of the filtered aqueous fraction generated during digestion of 2% milk containing NBX with Dulbecco's minimal essential medium supplemented with 1% L-glutamine and 1% nonessential amino acids. The test medium was incubated at 37 °C for 15 min before addition to cultures. Transport experiments were performed 21–25 days after monolayers grown on membrane inserts reached confluency (17). The extended period of incubation for monolayers on inserts is required for maximal secretion of chylomicrons during prandial-like culture conditions (18, 19). To study the transfer of NBX from the apical to the basolateral compartment, the carotenoid was prepared in medium containing mixed micelles with a physiologically relevant composition (17) instead of an aqueous fraction generated during simulated digestion. Medium added to the apical compartment (2 mL) contained 1.8 μ g of NBX. The integrity of monolayers on inserts was determined by inclusion of 500 μ M phenol red in apical medium and phenol red-free medium in the basolateral compartment to measure paracellular flux of the dye (16). The mean flux of phenol red flux was $0.02 \pm 0.005\%$ h⁻¹(cm²)⁻¹ in the presence and absence of NBX at tested concentrations. Exposure of cell monolayers to as much as 17 μ g/mL NBX did not significantly affect gross morphology of the monolayer, cell viability as assessed by sulforhodamine B (20), or cell protein content per well.

Extraction of NBX from Enriched Milk, Digesta, and Aqueous Fractions

Thawed aliquots (0.5–1 mL) of test food, digesta, and aqueous fractions were transferred to 15 mL polypropylene test tubes. An aliquot (100 μ L) of bixin (>99% pure as determined by HPLC and containing 88.5% 9'-*cis*-isomer) in acidified methanol was added as the internal standard (IS) to quantify extraction efficiency. Cell pellets were resuspended in 1 mL of ice-cold phosphate-buffered saline and sonicated twice for 5 s (Vibra Cell, Sonic Materials, Inc., Danbury, CT). Samples containing relatively high and moderate amounts of NBX required the addition of 1 mL of 0.05% acetic acid in water for efficient extraction of NBX, whereas 350 μ L of the acidic solution was appropriate for the remaining samples. Ethyl acetate (3 mL) was added, and the mixtures were mixed by vortex for 1 min at 2500 rpm in a Multitube Vortexer (model DVX-2500, VWR International, West Chester, PA). Tubes were centrifuged for 5 min at 3800g (model 2275 Centrifuge, Fisher Scientific Co.). The organic layer was transferred to a glass vial, the extraction procedure was repeated twice, and organic layers were pooled. Finally, 3 mL of 1:1:1 ethyl acetate:methanol:petroleum ether (1:1:1) was added to the pooled extract. The solvent was removed by flushing with N₂ and stored at -20 °C for no longer than 3 days before analysis. Samples were resolubilized in 1 mL of 2% acetic acid in methanol (v/v) and filtered (Anotop 10 inorganic membrane, 0.2 μ m pores; Whatman, Maidstone, England). The efficiency of extraction of NBX was assessed by recovery of 9-*cis*-bixin IS using the extinction coefficient of $E_{1\text{ cm}, 1\%} = 3130$ at 474 nm in 3.7% acetic acid in chloroform (v:v).

HPLC Chromatographic Analysis

The HPLC method was a modification of that described by Scotter et al. (14). A Waters HPLC 2690 Separation Module (Milford, MA) coupled to a 2996 Photodiode Array Detector was used. Separation of NBX isomers was achieved at 29 °C with a Waters NovaPak-C18 Column (3.9 mm × 150 mm, 4 μm) preceded by a guard column containing the same packing. The binary mobile phase consisted of an aqueous solution of 1% acetic acid (A) and 100% acetonitrile (B). NBX isomers were eluted with a flow rate of 1 mL/min and the following solvent gradient: 0–14 min, 65% B; 14–18 min, 90% B; 18–20 min, 65% B; and equilibration at 65% B from 20 to 25 min prior to the analysis of the next sample. The chromatograms were monitored from 300 to 600 nm, and peaks were integrated at 461 nm using Empower software (Waters). Peaks containing NBX isomers were identified by comparing spectral characteristics with the 9'-cis standard and available literature. The total NBX was quantitatively estimated by addition of the areas under the curve (AUCs) for isomers and comparison to peak areas from external calibration curve using known concentrations of 9'-cis-NBX (97.1% purity) and $E_{1\text{ cm}, 1\%} = 2850$ as the extinction coefficient.

Cell Protein

The quantity of cell protein in wells was determined using the bichinchoninic acid assay (Thermo Scientific Pierce, Rockford, IL) with bovine serum albumin as the standard as instructed by the manufacturer.

Statistical Analysis

Independent experiments with multiple replicates for in vitro digestion, cellular uptake, and cellular transport were performed at least twice ($n = 3-6$). Statistical analyses of data were performed using the SPSS statistical analysis software (16.0). All data are expressed as means ± standard errors of the mean (SEMs). Significant differences between samples were assessed using one-way analysis of variance followed by Tukey's posthoc test. $P < 0.05$ was set to define significant differences among samples.

RESULTS

Stability of NBX during Simulated Digestion

Isomers detected in the enriched milk included 9'-cis-NBX, 9',13'-di-cis-NBX, and all-trans-NBX, with traces of 13'-cis-NBX (Figure 1 and Table 1), in agreement with previous observations (8). Because of insufficient resolution of the peaks corresponding to the 9',13'-di-cis- and the all-trans-isomers in the chromatogram (Figure 2), the combined AUC for these two isomers is presented in Table 1 as a single percentage and henceforth referred to as all-trans-NBX. The recovery of NBX added to skim milk, 2% fat milk, and whole milk after simulated gastric and small intestinal digestion exceeded 96% and was independent of both the range of NBX concentrations and the fat content of milk (data not shown). During digestion, the relative amount of 9'-cis-NBX decreased from approximately 90 to 76–85% of total NBX with increased ($p < 0.05$) amounts of both all-trans- and 15'-cis-NBX noted (Table 1). The relative amount of all-trans-NBX was greater ($p < 0.05$) in digesta and the aqueous fraction for NBX-enriched skim milk as compared to that for digested 2% fat milk and whole milk.

Approximately 60% of total NBX added to milk partitioned into the filtered aqueous fraction during small intestinal phase of digestion and was independent of the fat content of the milk vehicle (Figure 3). The absence of bile extract during the small intestinal phase of digestion significantly decreased partitioning of NBX in the filtered aqueous fraction of

digesta. However, it is noteworthy that 30.1, 11.5, and 8.3% of total NBX were present in the aqueous fraction after digestion of NBX-enriched skim milk, 2% fat milk, and whole milk, respectively. Collectively, the results suggest that NBX partitions both within and external to mixed micelles.

Uptake and Transport of NBX by Caco-2 Cells

The filtered aqueous fraction generated during digestion of NBX-enriched milk containing 2% fat was diluted with Dulbecco's minimal essential medium and added to cultures to examine the uptake of NBX by differentiated cultures of Caco-2 cells attached to the plastic dish surface. The uptake of NBX from medium was proportional ($R^2 = 0.85$) to the concentration of the pigment in the medium and represented 6–8% of the initial amount of NBX added to cell cultures (Figure 4A). The cellular content of NBX slightly increased ($p < 0.05$) from 35 ± 1.1 ng/mg protein at 60 min to a maximum of 42 ± 1.8 ng/mg protein at 120 min during incubation in medium containing $1.85 \mu\text{g}$ of NBX (Figure 4B).

Caco cells were next grown and differentiated on membrane inserts to determine the extent of transport of NBX from the apical compartment with $1.80 \mu\text{g}$ of NBX to the basolateral compartment. The quantities of NBX in basolateral medium were 113 ± 2.2 (4.1% of total in well) and 124 ± 2.0 ng (4.6% of total in well) after 120 and 240 min, respectively. The cellular content of NBX was 44–51 ng/mg protein after incubation with apical medium-containing micelles and $1.8 \mu\text{g}$ of NBX for 60–240 min. The recovery of total NBX in apical and basolateral media plus the cell monolayer after 240 min was $72 \pm 1.3\%$ of the initial content. Two unidentified peaks with maximum absorbances at 424 and 449 nm, respectively, and retention times less than that for NBX isomers were present in the apical and basolateral media of cultures exposed to NBX at 240 min. This suggested that metabolism of a portion of NBX accounted for some of the apparent loss of the added compound during the incubation.

The relative amount of 9'-*cis*-NBX decreased ($p < 0.05$) from 85 to 70% within 15 min of diluting the aqueous fraction generated during simulated digestion (Figure 5A). By 240 min of incubation, 9'-*cis*-NBX declined further ($p < 0.05$), accounting for only 7% of total NBX in the apical medium. This was associated with all-*trans*-NBX becoming the predominant isomer ($p < 0.05$) and the relative amount of 15'-*cis*-NBX increasing from 2 to 5% ($p < 0.05$). Similar changes in the isomeric profile of NBX in medium were observed in wells with and without Caco-2 cells, suggesting that the isomerization was abiotic. In contrast with changes in the isomeric profile of NBX in serum-free medium, the intracellular profile of 9'-*cis*- (49–61% of total) and all-*trans*- (24–26% of total) NBX remained relatively constant ($p > 0.05$) from 30 to 240 min (Figure 5B). However, the relative amount of 15'-*cis*-NBX in cells increased ($p < 0.05$) from 12 to 26% ($p < 0.05$) from 30 to 240 min during incubation. These observations suggest possible association of NBX isomers with cellular protein, thus limiting isomerization. Within baso-lateral medium, the relative amounts of all-*trans*-NBX (40–50%) and 15'-*cis*-NBX (11–14%) in the basolateral compartment were not significantly ($p > 0.05$) altered as the period of exposure of cell to NBX increased and 9'-*cis*-NBX slightly declined ($p < 0.05$) between 30 and 240 min (Figure 5C).

DISCUSSION

The above results show that NBX is relatively stable during simulated gastric and small intestinal digestion and accessible for uptake and *trans*-epithelial transport across monolayers of polarized Caco-2 cells. This supports the likelihood that at least a portion of ingested NBX is bioavailable. The recovery of NBX after simulated digestion of the milk vehicle was high as previously reported for C₄₀ carotenoids in various food matrices (15, 17, 21, 22). 40-Carbon carotenoids, like other lipophilic dietary compounds and

pharmaceuticals, must partition into mixed bile salt micelles during digestion for delivery to the brush border membrane of enterocytes in the small intestine. The presence of bile salts during the small intestinal phase of digestion was associated with the transfer of approximately 60% of NBX in milk to the filtered aqueous phase of digesta. This extent of partitioning in the aqueous fraction was greater than that typically reported for 40-carbon carotenoids such as β -carotene, lutein, and lycopene during in vitro digestion of fruits and vegetables (12, 17, 21). It is interesting that 10–30% of NBX in milk partitioned in the aqueous fraction during simulated digestion in the absence of bile salts, whereas less than 5% of 40-carbon carotenoids is present in this fraction under similar conditions (13, 22). There are two likely factors that contribute to the relatively high bioaccessibility of NBX. First, commercial NBX added to foods has been isolated from annatto seeds, thereby eliminating the need to release the carotenoid from chromoplasts or chloroplasts during the digestion process. Second, NBX with its shorter hydrocarbon chain and carboxylate groups at both termini is more hydrophilic than 40-carbon hydrocarbon and oxy-carotenoids. This likely is the basis for the partitioning of NBX both within and external to micelles in the aqueous fraction of digesta. The impact of food matrix for commercial products containing annatto on NBX bioaccessibility was not examined here but certainly merits investigation.

Dietary fat is a well-established promoter of the absorption of 40-carbon carotenoids and other lipophilic compounds (11). The efficiency of micellarization during simulated digestion in the presence of bile salts was not affected by the fat content of the milk vehicle. Micellarization of 40-carbon carotenoids requires coingestion of a relatively low amount of triglyceride (23). However, additional fat is required for the assembly and secretion of chylomicrometers to transfer carotenoids from enterocytes to the periphery (11). For example, the bioavailability of carotenoids from vegetable salads was significantly greater when human subjects consumed a full fat dressing (28 g) as compared to reduced (6 g) and no fat dressings (24). We observed that approximately 4% of NBX present in apical medium along with lipid digestion products from milk with 2% fat was transported into the basolateral compartment during the 4 h incubation. The effect of substituting skim milk or whole milk for milk with 2% fat as the delivery vehicle on NBX transport across the Caco-2 monolayer was not examined. More detailed studies are needed to investigate chylomicron-dependent and -independent pathways of NBX absorption and the impact of types and amounts of fat on such processes.

Because global gene expression and the phenotype of differentiated cultures of Caco-2 human intestinal cells are quite similar to mature enterocytes (25–27), this cell line is widely used to investigate the uptake, metabolism, and *trans*-epithelial transport of diverse dietary compounds including carotenoids (13). Accumulation of NBX by Caco-2 cells was proportional to its concentration in medium and reached steady state as early as 30 min after initial exposure. As NBX is an anion at neutral pH, its transfer across the brush border membrane is likely facilitated by a transport protein. The uptake of β -carotene, lutein, lycopene, and vitamin E across the brush border membrane is mediated in part by scavenger receptor class B type 1 protein (28–31). The potential roles of scavenger receptor class B type 1, long-chain fatty acid transport proteins, and anion transport proteins in the transport of NBX and other short-chain carotenoids require investigation.

Information regarding in vivo absorption of NBX is limited. Both NBX and bixin, a monomethylated analogue of NBX (32), were present in plasma of humans orally administered 16 mg of bixin. The appearance of NBX in plasma was delayed as compared to bixin, suggesting that demethylation of bixin is mediated by gut bacteria or occurs postabsorption. The absorption of crocetin, a 20-carbon carotenoid also with carboxylate groups at the termini (Figure 1), has been investigated in fasted mice. The absorption of crocetin was rapid with both free crocetin and its glucuronide conjugates appearing in

plasma (33). Moreover, crocetin absorption occurred in the absence of coingested fat, suggesting *trans*-epithelial transport by a chylomicrometer-independent pathway. The possibility that NBX is partially metabolized to phase II conjugates by Caco-2 cells is supported by the appearance of polar molecules with spectral characteristics that are similar to the pigment in both apical and basolateral media, as well as the observation that cellular accumulation of NBX was maximum during the initial 30–60 min of exposure to the compound.

Finally, almost 90% of 9'-*cis*-NBX spontaneously isomerized to the all-*trans*- and the 15'-*cis*-isomers during the 4 h incubation in protein-free medium at 37 °C. Levy et al. (32) reported the presence of both all-*trans*-NBX and 9'-*cis*-NBX isomers in human plasma after ingesting 9'-*cis*-bixin. We observed that the isomeric profile of NBX in Caco-2 cells and in basolateral medium remained somewhat constant during incubation. Galindo-Cuspinera et al. (9) reported greater stability of NBX bound to proteins as compared to that in a protein-free solution. Our results suggest that enterocytes are able to take up both 9'-*cis*- and all-*trans*-isomers from the gastrointestinal lumen and that NBX isomers bind to proteins within these cells and are associated with lipoproteins or other proteins following transfer across the basolateral membrane. Further studies are now needed to systematically characterize the cellular metabolism, mechanism of absorption, and plasma distribution of NBX isomers.

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Figure 1.

Chemical structure of natural apo-carotenoids: *all-trans* crocetin and *all-trans*- and *9'-cis*-NBX ($R = H$). Bixin (BXN) is the monomethylated ($R = CH_3$) derivative of NBX that is also present in annatto.

Figure 2.

Representative HPLC chromatograms of NBX-enriched milk with 2% fat, digesta, and filtered aqueous fraction. NBX isomers detected in milk were (a) all-*trans*, (b) 9',13'-di-*cis*, (c) 9'-*cis*, and (d) 15'-*cis* as identified by Scotter et al. (8). IS, 9'-*cis* bixin, was added to NBX-enriched milk as the IS to assess extraction efficiency. NBX isomers and 9'-*cis*-bixin were eluted from the C-18 column using a binary gradient consisting of 1% acetic acid and 100% acetonitrile with detection at 461 nm as described in the Materials and Methods.

Figure 3.

Bile extract increases partitioning of NBX into aqueous fraction during simulated digestion of enriched milk. Data are means $\pm$ SEMs, $n = 3$ independent experiments. Different letters above the error bars indicate that the percentage of NBX in the aqueous fraction of digested milk was affected by presence of bile extract and fat content ($p < 0.05$).

Figure 4. Uptake of NBX by Caco-2 cells is proportional to the concentration of NBX in apical medium (**A**) but relatively independent of incubation period (**B**). Data are means $\pm$ SEMs, $n = 6$ independent experiments. Different letters above error bars indicate that means differ significantly ($p < 0.05$).

Figure 5.

Profile of NBX isomers added in apical medium during incubation at 37 °C in absence and presence of Caco-2 cells, in Caco-2 cells, and transported into basolateral medium. Data are means $\pm$ SEMs, $n = 3$ independent experiments. Different letters above error bars for a specific isomer indicate that there was a significant ($p < 0.05$) change in its relative abundance with time of incubation.

Table 1

Profile of NBX Isomers in Enriched Milk and Digesta and Aqueous Fraction after Simulated Gastric and Small Intestinal Digestion with Bile Salts^a

		NBX isomers (%) NBX		
	fat content	all- <i>trans</i> (+di- <i>cis</i>)	9'- <i>cis</i>	15'- <i>cis</i>
milk	skim	8.8 ± 0.2 b	91.2 ± 0.2 a	ND b
	2%	9.3 ± 0.3 b	90.7 ± 0.3 a	ND b
	whole	8.5 ± 0.3 b	91.6 ± 0.4 a	ND b
digesta	skim	21.6 ± 0.3 a	75.5 ± 0.4 b	2.8 ± 0.1 a
	2%	16.3 ± 0.9 a	80.6 ± 1.0 b	3.2 ± 0.1 a
	whole	13.3 ± 0.4 a	85.2 ± 0.5 b	2.3 ± 0.2 a
aqueous fraction	skim	20.4 ± 0.2 a	75.8 ± 0.2 b	3.7 ± 0.1 a
	2%	18.6 ± 0.5 a	77.4 ± 0.5 b	3.9 ± 0.1 a
	whole	14.8 ± 0.9 a	81.9 ± 0.7 b	3.3 ± 0.2 a

^aData are means ± SEMs for $n = 3$ independent experiments. Different letters within a row indicate that the percentage of the NBX isomer in the specific milk significantly differs ($p < 0.05$) from that in total digesta and its aqueous fraction.