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Short communication: Development of a novel method for the extraction of norbixin from whey and its subsequent quantification via high performance liquid chromatography

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ABSTRACT

Norbixin is the primary carotenoid in annatto coloring, which imparts the desired orange color in Cheddar cheese. However, a portion of the colorant remains in the cheese whey and is undesirable; therefore, a bleaching step is often applied. Restrictions exist for norbixin concentrations in products destined for infant formula. As such, evaluation of norbixin concentrations in whey and whey ingredients is desirable. Current extraction methods are laborious and require solvents that are banned in many countries. The objective of this study was to develop a fast and inexpensive norbixin extraction and quantitation technique using approved solvents with similar sensitivity to current established methods. Instead of solvent extraction and column purification, acetonitrile was added directly to fluid wheys, retentates, and rehydrated whey protein concentrates. An isocratic mobile phase [70% acetonitrile and 30% water with 0.1% (wt/vol) formic acid] was used and, to increase sensitivity, a large volume (50 μL) was injected onto the column. The column used was a C18 column with a particle size of 2.6 μm and column length of 10 cm. The column inner diameter was 4.6 mm and the pore size was 100 Å. All of the previously described conditions allowed the run time to be only 4 min. The sample was sent through a photodiode array detector and quantified at 482 nm. Norbixin was quantified using external standard curves. The developed method had a >90% norbixin recovery in both milk and whey (9.39 μg/L–2.35 mg/L). The limit of detection of norbixin in fluid whey was 2.7 μg/kg and the limit of quantitation was 3.5 μg/kg , both of which are significantly lower than in previously described methods. The extracts were stable over 30 min at 21°C and stable over 24 h at 4°C. Repeatability and precision of the method had relative standard deviations of less than 13%. The developed method provides time and cost savings for evaluation of norbixin concentration in whey and whey products.
Key words: norbixin, annatto, whey, HPLC

Short Communication

Cheddar cheese is often colored with annatto, a yellow-orange carotenoid primarily composed of norbixin, to impart the desired orange color (Kang et al., 2010). A portion of the colorant remains in the cheese whey and is undesirable (Kang et al., 2010). To remove the color, a bleaching step is often applied. In the United States, 2 chemicals are approved for the bleaching of whey: hydrogen peroxide and benzoyl peroxide. Due to increasing regulations regarding these 2 bleaching agents, various alternative bleaching agents have been investigated (Campbell et al., 2012; Kang et al., 2012). In addition, legal limits for norbixin concentrations also exist in products destined for infant formula. As such, evaluation of norbixin concentrations in whey and whey ingredients is desirable.

High performance liquid chromatography is a highly sensitive technique used to separate and subsequently quantify nonvolatile components. Carotenoids are often measured using a photodiode array detector, which measures absorbance of eluent components at a certain wavelength. The maximum wavelength for norbixin absorbance is 482 nm (Kovary et al., 2001). As HPLC does not require large sample volumes, it is a very versatile technique that can be applied to a wide array of products, from food to pharmaceuticals to environmental contaminants.

Current extraction methods for norbixin are time intensive, laborious, and expensive (Lancaster and Lawrence, 1995; Scotter et al., 2002; Croissant et al., 2009; Table 1). In addition, current extraction methods require solvents that are banned in many countries. The objective of this study was to develop a fast and inexpensive extraction technique using approved solvents with similar sensitivity to current established methods.

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Table 1. Cost and time analysis of various norbixin (annatto) extraction methods (prices calculated using Fisher Scientific, Waltham, MA,as the supplier of materials)

Item	Cost ($)	Extraction time (h, unless otherwise noted)	Waste (mL)
Scotter et al. (2002)			
Solvent			
100 mL of hexane	12.04		
3 g of celite	0.43		
3 mL of 0.1% BHT (in methanol)	0.05		
40 mL of ethanol:water:ammonia (100:35:15)	0.58		
50 mL of acetic acid (11%)	3.61		
25 mL of methanol	1.34		
75 mL of chloroform:acetic acid (98.5:1.5)	9.73		
Disposable goods			
Glass wool	1.47		
Total	27.78	2.5	293
Lancaster and Lawrence (1995)			
Solvent			
3 g of celite	0.43		
75 mL of ethanol:water:ammonium hydroxide (100:35:15)	1.09		
100 mL of petroleum ether	11.4		
100 mL of acetic acid (11%)	7.22		
120 mL of chloroform:glacial acetic acid (98.5:1.5)	15.57		
10 mL of choloroform	1.3		
55 mL of methanol	3.81		
Disposable goods			
Glass wool (×2)	2.94		
Total	40.82	2.5	460
Croissant et al. (2009)			
Solvent			
6 mL of ethanol	0.23		
6 mL of chloroform	0.78		
2 mL of acetic acid (1%)	0.07		
7 mL of hexane	0.84		
5 mL of hexane:diethyl ether (1:1)	0.59		
1 mL of acetone	0.11		
3 mL of methanol:glacial acetic acid (7:3)	0.21		
Disposable goods			
SPE Strata NH2 column[1]	4.08		
Total	6.91	2	30
Proposed method			
24 mL of acetonitrile:water (80:20)	3.21		
Total	3.21	15 min	24

[1]Solid-phase extraction Strata NH2 column (Phenomenex, Torrance, CA).

Linearity, stability, and repeatability were measured to confirm that the new extraction and quantification method were equivalent or better than current methods for these items.

Cheddar whey was manufactured from vat-pasteurized whole bovine milk (195 kg) as described by Campbell et al. (2011). To color the milk, annatto was added at a rate of 15 mL/454 kg of milk (Danisco, St. Louis, MO; 3% norbixin wt/vol). The whey was drained from the curds at pH 6.35 and a sieve was used to remove any remaining particles. The whey was immediately processed with a hot bowl cream separator (Model SI600E, Agri-Lac, Miami, FL) to reduce the fat content. The whey was pasteurized at 63°C for 30 min. A portion of fluid whey was removed for bleaching. Two different bleaching treatments were applied: hydrogen peroxide (**HP**) and lactoperoxidase. For HP chemical bleaching, 250 mg/kg of HP was added to liquid whey and allowed to react for 1 h at 50°C with gentle agitation. The concentration of HP was selected because it represents the midrange of the legally allowed amount of HP for traditional chemical bleaching of whey and also represents a concentration that is generally applied by industry (Kang et al., 2010; Listiyani et al., 2011). Catalase (20 mg/kg, FoodPro CAT, Danisco, New Century, NJ) was added at a rate of 20 mg/kg to remove excess HP and stop the bleaching process. For lactoperoxidase, 20 mg/kg of HP was added to fluid whey to activate the enzymatic system and allowed to bleach for 1 h at 50°C with gentle agitation. Catalase was added to remove any excess HP and stop the enzymatic bleaching process.