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Fast cleavage of phycocyanobilin from phycocyanin for use in food colouring

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ABSTRACT

Phycocyanins from cyanobacteria are possible sources for new natural blue colourants. Their chromophore, phycocyanobilin (PCB), was cleaved from the apoprotein by solvolysis in alcohols and alcoholic aqueous solutions. In all cases two PCB isomers were obtained, while different solvent adducts were formed upon the use of different reagents. The reaction is believed to take place via two competing pathways, a concerted E2 elimination and a S_N2 nucleophilic substitution. Three cleavage methods were compared in terms of yield and purity: conventional reflux, sealed vessel heated in an oil bath, and microwave assisted reaction. The sealed vessel method is a new approach for fast cleavage of PCB from phycocyanin and gave at 120 °C the same yield within 30 min compared to 16 h by the conventional reflux method ($P < 0.05$). In addition the sealed vessel method resulted in improved purity compared to the other methods. Microwave irradiation increased product degradation.

1. Introduction

The growing demand for more natural food products is pushing the food and beverage industry towards the replacement of synthetic colourants. This trend increased after the publication of studies that linked the consumption of artificial colourants and additives with behavioural changes in children (McCann et al., 2007; Rowe & Rowe, 1994). Natural colourants are generally more accepted. However, their incorporation is still a challenge for food technologists, as they are typically less vivid, less stable, and more expensive than their synthetic counterparts (Wrolstad & Culver, 2012). Regarding blue colour, the most widespread synthetic colourant is Brilliant Blue FCF (E133), also referred to as FD & C Blue No. 1. The natural alternative to Brilliant Blue FCF did not come straight forward, as blue coloured compounds are relatively rare in nature. Newsome, Culver, and Van Breemen (2014) provided a review of blue pigments found in animals, plants, fungi, and microbes, and concluded that none of them seemed likely to match all the criteria of shade, brilliance, and stability of Brilliant Blue FCF, while at the same time meeting the requirements of safety, abundance, and economic viability. However, Jespersen, Strømdahl, Olsen, and Skibsted (2005) compared three natural blue colourants: gardenia blue, phycocyanin, and indigo, in terms of stability in different food applications, and concluded that although none of them were ideal, phycocyanin was the most versatile.

The phycocyanin-based colourant Spirulina extract was recently approved by the U.S. Food & Drug Administration as colour additive exempt from certification (Spirulina extract. In., 2013). Its main components, the phycocyanins C-phycocyanin (C-PC) and allophycocyanin (APC), are two of the photosynthetically active proteins common in cyanobacteria (O'hEocha, 1965). Their biological role is to enhance the absorption of visible light into the range where chlorophyll *a* absorbs poorly (Croce & Van Amerongen, 2014). As they absorb in the range of the spectrum corresponding to the red light (~600 nm), they are perceived as blue. However, phycocyanin colourants still deal with some challenges regarding their poor stability to light, heat and acid matrices (Jespersen et al., 2005; Moreira et al., 2012).

The blue colour of these proteins is attributed to the chromophore phycocyanobilin (PCB), an open-chain tetrapyrrole which is covalently bound to the polypeptide chains through thioether bonds at selected cysteine residues (Bishop et al., 1986). Some of the disadvantages of phycocyanin like precipitation in acid matrices and bleaching due to denaturation by heat are related to their protein nature. Therefore it is hypothesized that cleavage of PCB from the proteins and further stabilization of the molecule could be a possible approach to help solve the stability issues mentioned above.

The PCB chromophore can be cleaved from the apoprotein by different methods including acid cleavage, enzymatic treatment or methanolysis (Beuhler, Pierce, Friedman, & Siegelman, 1976; Chapman,

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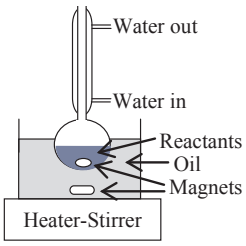
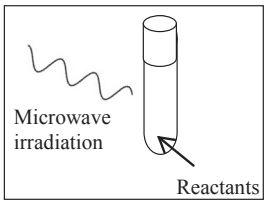
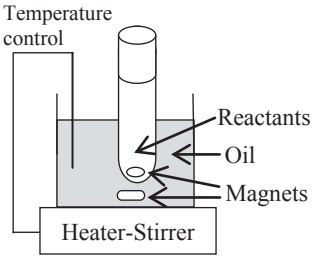
Table 1
Experimental outline. Different cleavage methods, schematic representation and parameters used.

Parameters tested	Cleavage methods Schematic representation		
	Conventional reflux	Microwave assisted	Sealed vessel
		 Microwave digestion system	
Reagents	Methanol, ethanol, ethanol 96% and ethanol 70%	Ethanol 96%	Ethanol 96%
Reaction time	16 h	5, 15, 30, 60 and 90 min	5, 15, 30, 60, 90 min
Temperature	Boiling point of each solvent	90, 100, 120 and 150 °C	90, 100, 120 and 150 °C
Phycocyanin-solvent ratio	100 ml g^{-1}	100 ml g^{-1}	50, 100 and 150 ml g^{-1}

Cole, & Siegelman, 1968).

The use of microwave irradiation to accelerate organic reactions is becoming increasingly popular among chemists (Adam, 2003). Especially after the development of modern laboratory microwave ovens with reliable pressure and temperature controls, that ease the reproducibility and understanding of the processes. Microwave irradiation provides a much faster heating and a more homogenous temperature distribution of reaction mixtures as compared to oil-bath heating (Kappe, 2004). In most cases the rate enhancements are attributed to the higher heating rate, which is referred to as the thermal/kinetic effect. However, some "specific microwave effects" that cannot be achieved by conventional heating have also been reported, and are still subject of debate (Perreux & Loupy, 2001).

In the present work, cleavage of PCB from a mixture of phycocyanins from the cyanobacterium *Arthrospira platensis* (formerly named *Spirulina platensis*) has been achieved by the means of solvolysis in alcohols at high temperatures. The methods conventional heating under reflux, heating in a sealed vessel in an oil bath, and microwave assisted cleavage were compared in terms of yield and purity of the product mixtures (see Table 1). Separation and identification of the released pigments were performed by high-performance liquid chromatography (HPLC), liquid chromatography–mass spectrometry (LC–MS) and/or NMR spectroscopy.

2. Materials and methods

2.1. Phycocyanin-based colourant and chemicals

The phycocyanin-based colourant Linablue® G1 was acquired from DIC Europe GmbH (Düsseldorf, Germany). Methanol, acetonitrile, absolute ethanol, ethanol 96% and 70% v/v were acquired from Sigma Aldrich (Brøndby, Denmark). Reagent grade formic acid and trifluoroacetic acid were obtained from Sigma-Aldrich. Deionised water was used for all aqueous solutions and dilutions (Purelab Chorus, Krüger Aquacare, Ninolab, Solrød Strand, Denmark).

2.2. Preparation of denatured phycocyanin

The commercial food colourant Linablue G1® was used as source of phycocyanin. Its absorption spectrum at pH 7 is shown in Fig. 1. Linablue G1® contains 21.5 ± 0.3% w/w C-phycocyanin and 6.2 ± 0.1% w/w allophycocyanin, as found by the spectrophotometric method of Yoshikawa and Belay (2008). Its main additive, D-trehalose, was removed by successive washes with methanol, which also caused protein denaturation. 50 g of Linablue G1® were mixed with 400 ml

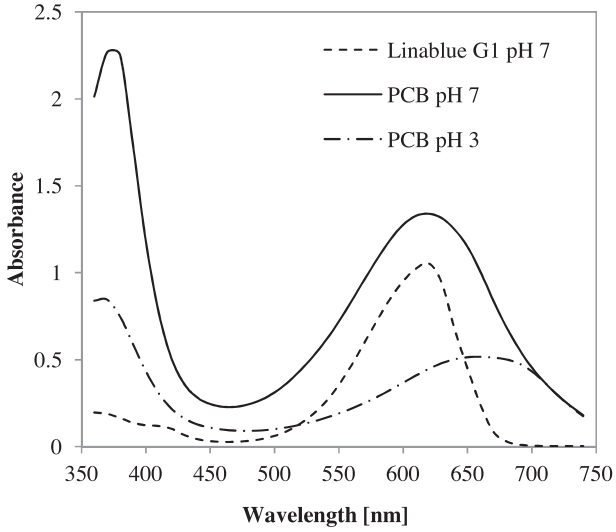


Fig. 1. Visible absorption spectrum of Linablue G1® at pH 7 and of free PCB at pH 7 and 3.

methanol and stirred at room temperature for 20 min. The suspension was filtered through a glass filter type 4 Whatman TM, Spartan 13/0.2 µm RC (VWR, Søborg, Denmark). The cake containing the phycocyanin was recovered and the liquid phase was analysed. The wash process was repeated until no more D-trehalose was detected in the liquid phase by HPLC equipped with a refractive index detector. The stationary phase was a Rezex™ RHM-Monosaccharide H+ (8%), LC column (300 × 7.8 mm, 8 µm), (Phenomenex, Værløse, Denmark) and the mobile phase deionized H_2O at a flowrate of 0.6 ml min^{-1}. The temperatures of the column compartment and the detector were set to 80 °C and 60 °C, respectively. The final phycocyanin cake was dried in a fume hood at room temperature until no further loss of weight was observed.

2.3. Cleavage methods

The experimental outline including a schematic representation of the different cleavage methods is detailed in Table 1.

2.3.1. Conventional reflux cleavage

1.0 g of dried phycocyanin cake was mixed with 100 ml of reagent in a 200 ml round bottom flask paired with a reflux condenser. The flask was equipped with a magnetic stirrer and submerged in a silicon oil bath heated to a temperature 10 °C above the boiling point of the reagent. The stirring velocity was 500 rpm. Four different reagents were