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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 hours 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.

Keywords: Natural blue colourant, Chromophore, Methanolysis, Solvolysis, Reaction mechanism

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 some studies linking the consumption of artificial colourants and additives with behavioural changes in children (McCann, Barrett, Cooper, Crumpler, Dalen, Grimshaw, 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," 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, Strømdahl, Olsen, & Skibsted, 2005; Moreira, Passos, Chiapinni, Silveira, Souza, Coca-Vellarde, et al., 2012).

The blue colour of these proteins is attributed to the chromophore phycocyanobilin (PCB), an open-chain tetapyrrole which is covalently bound to the polypeptide chains through thioether bonds at selected cysteine residues (Bishop, Lagarias, Nagy, Schoenleber, Rapoport, Klotz, 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, 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 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 x 7.8 mm, 8 µm), (Phenomenex, Værløse, Denmark) and the mobile phase deionized H2O 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 tested with this method: methanol, ethanol, ethanol 96% and 70% v/v. Running tap water was used to cool the condenser.

In preliminary studies, samples of the product mixture obtained with the four different solvents were taken at one hour time intervals, and analysed by HPLC. The concentration of products was found to be highest after 16 hours; therefore this reaction time was selected for the set of experiments.

The conventional reflux reaction was stopped after 16 hours by submerging the flask in an ice bath for 15 min. The reaction mixture was filtered through a glass filter type 4 Whatman TM, Spartan 13/0.2 µm RC. The deep blue solution was collected and analysed by HPLC, while the green solid protein residue was discarded. Determination of dry matter in the solution was done by evaporation in a rotary evaporator R-210 (Buchi, Flawil, Switzerland).
In the case of ethanol 70%, the remaining water was removed with a freeze dryer (CoolSafeTM, Scanvac, Ninolab, Solrød Strand, Denmark).

2.3.2 Sealed vessel cleavage

100 mg of dried phycocyanin cake was mixed with 10 ml of ethanol 96% v/v in a pressure vessel HVT50 (Multiwave GO, Anton Paar GmbH, Graz, Austria) equipped with a magnetic stirrer. The stirring velocity was 500 rpm. The flask was placed in a silicon oil bath previously heated to the desired temperature and kept there for a specific amount of time. As the temperatures were above the normal boiling point, a build-up of pressure took place inside the vessel, which needed to be cooled down before opening and handled carefully. The reaction was stopped by submerging the flask in an ice bath for 15 min. The reaction mixture was filtered and the resulting deep blue solution was analysed. The dry mater content in the solution determined as described in section 2.2.1.

2.3.3. Microwave assisted cleavage

0.1 g of dried phycocyanin cake was mixed with 10 ml of solvent in a pressure vessel HVT50 (Multiwave GO, Anton Paar GmbH, Graz, Austria). Three vessels were loaded into a microwave digestion system (Multiwave GO, Anton Paar GmbH, Graz, Austria). Each run consisted of a 5 min heating ramp, kept at the target temperature for the desired amount of time and finally cooled down to 40 ºC during 15 min. The reaction was stopped by submerging the flask in an ice bath for 15 min. The reaction mixture was filtered and the resulting deep blue solution was analysed. The dry mater content in the solution determined as described in section 2.2.1.
2.4. Analyses of the product mixtures

2.4.1. Visible spectroscopy

The visible absorbance spectra were measured in a CM-5 spectrophotometer (Konica Minolta, Västrå Frölunda, Sweden) using 1 cm plastic cuvettes and 10 nm wavelength intervals.

2.4.2. Analytical chromatography

Chromatographic separation of the reaction products was performed by analytical HPLC equipped with a photodiode array (PDA) detector. HPLC chromatograms were recorded at 666 nm, coinciding with the visible maximum of PCB in acidic conditions (see Fig 1., PCB at pH 3). The reaction products were separated on a reversed phase C18 Eclipse (5 μm, 150 x 4.6 mm) column from Phenomenex (Værløse, Denmark). The mobile phase consisted of deionized H₂O containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The following gradient was used: 40% B at 0 min and 55% B at 15 min. The column temperature was 35°C, the flowrate was 0.8 ml min⁻¹, and the injection volume was 10 μl.

2.4.3. Liquid chromatography – mass spectrometry (LC-MS)

The same column and solvent gradient as in analytical chromatography (see Section 2.4.2.) were used for the LC–MS analysis of the reaction products. LC-MS analyses were performed on a LTQ XL (Thermo Scientific, Waltham, MA, USA) equipped with an Accela HPLC Pump and PDA detector. The HPLC separation was carried out as described above. The MS was run with the atmospheric pressure chemical ionization (APCI) in positive mode. Settings for the mass spectrometer were 50, 5 and 5 (arbitrary units) for sheath, auxiliary and
sweep gas flow rates, respectively, vaporizer temperature 450°C, discharge current 5 µA, capillary temperature 275°C, capillary voltage 45 V, tube lens 100 V.

2.4.4. Preparative chromatography

The main PCB isomer was isolated by preparative HPLC on a Dionex UltiMate 3000 Binary Semi-preparative LC System from Thermo Fisher Scientific (Hvidovre, Denmark), equipped with PDA, and a Foxy Jr. fraction collector unit from Teledyne ISCO Inc. (NE, USA). Separations were performed on a Develosil™ ODS-HG LC column (5µm, 250 x 20 mm) from Nomura Chemical Co. Ltd (Værløse, Denmark). The mobile phase consisted of deionized H₂O containing 0.05% trifluoroacetic acid (solvent A) and acetonitrile containing 0.05% trifluoroacetic acid (solvent B). The following gradient was used: 50% B at 0 min and 55% B at 40 min. The column temperature was 21°C, the flowrate 5 ml min⁻¹, and the injection volume was 2 ml, with a concentration in the sample of 4 mg ml⁻¹. The purity of the collected samples with regard to other isomers was assessed by analytical HPLC. It was calculated as the percentage of the area in the chromatogram of the main isomer, relative to the cumulative area of all compounds eluted. The fractions with chromatographic purity higher than 99.0 % were pooled together, evaporated in a speed-vac (CoolSafe™, Scanvac) and used to make a calibration curve for quantification purpose ($R^2 > 0.999$).

2.4.5. Nuclear magnetic resonance (NMR) spectroscopy

1D and 2D NMR spectra were run on a sample of the main isomer with chromatographic purity (99.0 %). The measurement was conducted at room temperature on a Bruker Avance 500 spectrometer in pyridine-$d_5$. The data were processed using MestReNova 6.0 software (Mestrelab Research SL, Santiago de Compostela, Spain).
The chemical structure of the main isomer (retention time ~ 4.8 min, Fig 2) was identified as (3E)-PCB based on 1D and 2D NMR. (3E)-PCB: $^1$H NMR (pyridine-$d_5$, 500 MHz): $\delta_H$ 1.28 (3H, $t$, $J = 7.5$ Hz, H$_3$-18”), 1.52 (3H, $d$, $J = 7.5$, H$_3$-2”), 1.72 (3H, $d$, $J = 7.2$, H$_3$-3”), 2.03 (3H, $s$, H$_3$-7”), 2.11 (3H, $s$, H$_3$-17”), 2.16 (3H, $s$, H$_3$-13”), 2.52 (2H, sept, $J = 7.5$, H$_2$-18”), 2.88 (2H, unresolved $t$ due to overlapping signal, H$_2$-8”), 2.89 (2H, unresolved $t$ due to overlapping signal, H$_2$-12”), 3.15 (2H, $t$, $J = 7.4$, H$_2$-8”), 3.23 (2H, $t$, $J = 7.2$, H$_2$-12”), 3.39 (1H, $br$ q, $J = 7.5$, H-2), 5.89 (1H, $s$, H-5), 6.10 (1H, $s$, H-15), 6.34 (1H, q of doublets, $J = 7.2$, 2.1, H-3”), 7.35 (1H, $s$, H-10). $^{13}$C NMR (pyridine-$d_5$, 125 MHz): $\delta_C$ 9.1 (C-13”), 9.4 (C-17”), 9.6 (C-7”), 13.3 (C-18”), 14.5 (C-3”), 15.9 (C-2”), 17.4 (C-18”), 20.5 (C-12”), 20.7 (C-8”), 36.6 (C-8”), 36.6 (C-12”), 38.2 (C-2), 87.7 (C-5), 96.2 (C-15), 112.7 (C-10), 122.6 (C-3”), 123.9 (C-13), 132.8 (C-12), 134.2 (C-18), 135.3 (C-14), 137.0 (C-7), 137.1 (C-3), 139.6 (C-17), 141.7 (C-11), 141.7 (C-16), 143.0 (C-8), 146.3 (C-4), 149.3 (C-9), 165.6 (C-6), 175.2 (C-19), 175.8 (C-12”), 176.0 (C-8”), 178.2 (C-1). Assignments of $^{13}$C NMR signals are based on HSQC. In addition the structure of (3E)-PCB was confirmed by $^1$H−$^1$H COSY and HMBC.

2.5. Analysis of the results

All experiments were performed in triplicate and the results are presented as mean ± standard deviation (error bars in the figures). Statistical significance was assessed through one-way analysis of variance (ANOVA) with significance limit $P < 0.05$. Data points labelled with the same letter in the figures are statistically equivalent.

Quantification of the PCB isomers was done by HPLC using the calibration curve built in section 2.4.4. The yield of the reactions was calculated as the quantified milligrams of the two PCB isomers obtained per unit gram of phycocyanin used as starting material, as detailed
by Eq. (1). The purity is expressed as the mass of PCB isomers per unit mass of dry raw product mixture, as shown in Eq. (2).

\[
Yield \ [mg \ g^{-1}] = \frac{mg \ (3E)PCB + mg \ (3Z)PCB}{g \ phycocyanin} \tag{1}
\]

\[
Purity \ [% \ w/w] = \frac{mg \ (3E)PCB + mg \ (3Z)PCB}{mg \ dry \ product \ mixture} \cdot 100\% \tag{2}
\]

3. Results and discussion

3.1. Conventional reflux cleavage

Boiling phycocyanins in methanol under reflux during long periods of time is the most widely used process to cleave PCB from phycocyanins. However, cleavage has also been reported by using hot butanol (Fu, Friedman, & Siegelman, 1979) and ethanol (O’Carra & O’hEocha, 1966), although in the latter only trace amounts of PCB were released. It is however expected that a solvolysis reaction as done with methanol and butanol will also take place with ethanol, since the functional group involved in the reaction is the alcohol moiety and not the hydrocarbon chain. In this study, the use of methanol, ethanol and two different aqueous ethanol solutions are shown to successfully cleave PCB from phycocyanins. However, different profiles of products, yields and purities were observed upon the use of different reagents.

3.1.1. Effect of the reagent on the profile of products

The chromatogram of the products obtained by conventional reflux of phycocyanin using the four reagents is shown in Fig. 2. The two main peaks found in all cases were regarded as PCB isomers, and have retention times of ~ 4.8 min and ~ 6.6 min, respectively.
Upon LC–MS analyses, both showed a quasi-molecular ion at \( m/z \) 587 [M + H]\(^+\). Fu et al. (1979) suggested these to be two cis-trans isomers of PCB, formed after the rotation of the central methine bridge single bond and stabilized by intra-molecular hydrogen bonding (Fu, Friedman, & Siegelman, 1979). However, further studies by Beale and Cornejo (1984, 1991) reported the two products to be related by isomerism at the double bond formed upon cleavage of the thioether linkage. They assigned the major methanolysis cleavage product to be (3E)-PCB, and the minor (3Z)-PCB, based on their visible absorption spectra, the fact that both could form the same ethylidene free derivative mesobiliverdin (Beale & Cornejo, 1984) and by comparison with the NMR spectra of other E/Z bilin isomers (Beale & Cornejo, 1991). Besides, they found both isomers were interconvertible, the (3E) isomer being more thermodynamically stable and hence the more abundant product in methanolysis reactions (Beale & Cornejo, 1984). Based on their mass spectra and ratio of abundance, the isomers eluting at ~ 4.8 and ~ 6.6 min were regarded as the (3E)- and (3Z)-PCB isomers, respectively.

The most abundant PCB isomer was isolated by preparative HPLC and its structure elucidated by NMR spectroscopy (see Materials and methods). The \(^1\)H NMR spectrum of this isomer clearly indicated that it was (3E)-PCB by comparison with literature values (Arciero, Dallas, & Glazer, 1988; Beale & Cornejo, 1991; Knipp, Müller, Metzler-Nolte, Balaban, Braslavsky, & Schaffner, 1998). The \(^1\)H NMR spectrum exhibited signals from six methyl groups (\( \delta_H \) 1.28, 1.52, 1.72, 2.03, 2.11, 2.16) of which three were singlets, five methylene (\( \delta_H \) 2.52, 2.88, 2.89, 3.15, 3.23) and five methine groups (\( \delta_H \) 3.39, 5.89, 6.10, 6.34, 7.35) in accordance with the tetrapyrrole structure of PCB. The chemical shifts of H-3’ at \( \delta_H \) 6.34 and H3-3’’ at \( \delta_H \) 1.72 confirmed the E-configuration of the PCB isomer (Beale & Cornejo, 1991). The \(^{13}\)C NMR spectrum also showed the presence of 17 sp\(^2\) quaternary carbons of which 15 were part of the five-membered heterocyclic ring structures of PCB, including two \( \gamma \)-lactam...
carbonyl carbons at δC 175.2 (C-19) and 178.2 (C-1). The two remaining sp² quaternary carbons at δC 175.8 (C-12′′′) and 176.0 (C-8′′′) were assigned to two carboxylic acid carbons. The position of all sp² quaternary carbons in PCB was confirmed by the HMBC spectrum. Finally the ¹H−¹H COSY spectrum revealed a long-range spin system of H-2/H-3′/H3-3′′ that clearly demonstrated an allylic coupling between H-2 (δH 3.39) and H-3′ and a homallylic coupling between H-2 and H3-3′′ as well as separate spin systems between H2-8′/H2-8′′, H2-12′/H2-12′′ and H2-18′/H3-8′′ in accordance with the chemical structure of PCB. Thus based on the above data it was concluded that the most abundant isomer formed after methanolysis was (3E)-PCB (Fig. 3).

The peak observed at retention time of ~ 4.1 min in the methanolic mixture is most likely a methanol adduct of phycocyanobilin (PCB-OMe), as confirmed by its quasi-molecular ion at m/z 619 [M+H]⁺. The formation of methanol adducts upon methanolysis has also been reported before in bibliography (Beuhler, Pierce, Friedman, & Siegelman, 1976). Similarly, an ethanol adduct of phycocyanobilin (PCB-OEt) was observed only in the ethanolic mixtures, with retention time 5.7 min as confirmed by its quasi-molecular ion at m/z 633 [M+H]⁺. Even though O’Carra et al. reported that only traces of PCB could be cleaved from phycocyanin in hot ethanol (O’Carra & O’hEocha, 1966), in the present work we have demonstrated that ethanol is also effective for this purpose.

3.1.2 Effect of the reagent on the yield and purity

As shown in Fig. 4., the reaction in methanol achieved a higher yield (17.2 ± 1.2 mg g⁻¹) than the reaction in ethanol (14.9 ± 0.3 mg g⁻¹), despite of having a lower boiling point (64.7 and 78.4°C for methanol and ethanol, respectively). This behaviour can be explained as
methanol, being a smaller molecule and having less steric hindrance, has more accessibility to the reaction centres and is hence a better nucleophile. The higher nucleophilicity of methanol can also account for the lower purity of the product mixture, as methanol will possibly be more reactive at other locations on the phycocyanins and release more by-products to the solution.

The yield of the methanolysis reaction obtained in this study cannot be directly compared to those reported earlier, due to the different expressions of yield used in the literature. Chapman et al. reported yields of PCB by methanolysis of 10.3 %, where the yield is expressed as the percentage of the absorbance of the free chromophore compared to that of denatured phycocyanin in neutral chloroform (Chapman, Cole, & Siegelman, 1968). It is now known, that the absorbance profile of the chromophore is highly dependent on its environment (Falk, 2012). Therefore it cannot be concluded that the absorbance profile of the free chromophore is necessarily equivalent to that of the denatured protein. Crespi et al. reported the yield of PCB as 40 – 50 % based on the ratios of absorbance at two different wavelengths of the protein and the chromophore (Crespi, Boucher, Norman, Katz, & Dougherty, 1967). In this study, the yield has been quantified as the mass in mg of the two PCB isomers per unit gram of phycocyanin used as starting material. However, as the methanolysis method remains identical as in the literature, the yield reported in this study can be expected to be the same as in the references mentioning methanolysis.

In Fig. 4 it is also shown how the use of aqueous ethanol solutions (96% and 70 % v/v) reached the same yield of products: 20.5 ± 0.4 mg g⁻¹ and 21 ± 2 mg g⁻¹, respectively. These yields were higher than in both ethanol and methanol. This effect is most likely due to changes in the protein structure caused by the presence of water. The water molecules solvate the hydrophilic residues of the protein, that can result in a partial unfolding of the still
denatured protein clusters. This phenomenon facilitates the accessibility of the reagent to the reaction centres. Structural changes of proteins in ethanol–water mixtures have been reported earlier, although typically for low ethanol concentrations (Ghosh, Roy, & Bagchi, 2013). The purity of the product mixtures was lower in solvents containing water. This trend was expected, as the amount of water soluble protein by-products increases with increasing proportions of water, as shown by the purities of 53 ± 2 % w/w, 38 ± 4 % w/w and 14 ± 2 % w/w, for ethanol, ethanol 96% and ethanol 70%, respectively. It can therefore be concluded that the presence of water in the solutions increases the yield of the reaction, while at the same time it lowers the purity of the product mixture. The choice of solvent is therefore a compromise between yield and purity. In the present study ethanol 96% v/v was the solvent of choice for further studies as it combines the highest yield (20.5 ± 0.4 mg g⁻¹) with a moderately high purity (38 ± 4 % w/w).

3.1.3. Effect of the reaction time on the yield
In all experiments using conventional reflux, and regardless of the solvent used, the reactions needed about 16 hours to achieve the yields reported. Longer reaction times did not result in increase of the yields, and therefore the conventional reflux reactions were stopped after 16 hours.

3.2. Sealed vessel and microwave assisted cleavage
3.2.1. Effect of temperature and microwave irradiation on the yield and purity
The use of sealed vessels allowed the increase of the reaction temperature above the boiling point of the reagent at atmospheric pressure. The incorporation of higher temperatures and microwave irradiation was implemented to attempt an increase in the rate and/or yield of
the reaction. The yields obtained for the two methods at four different temperatures using
ethanol 96% v/v (boiling point 78.2 °C) and a reaction time of 30 min are shown in Fig. 5A.
For comparison, the yield of the conventional reflux method (16 h) for the same reagent is
also shown. It is clearly observed how the use of higher temperatures resulted in increased
reaction rates, showing a marked thermal/kinetic effect. The optimal temperature for the
sealed vessel method was found to be 120 ºC, reaching the same yield (20.7 ± 0.7 mg g⁻¹) as
the conventional reflux method did in 16 hours for the same reagent. However, the yield
obtained by the conventional method could not be improved by increasing the temperature
further, showing that the maximum cleavage of PCB with this technique was already
achieved. At 150 ºC the yield obtained was slightly lower (19 ± 1 mg g⁻¹), showing that the
products started to degrade.

The optimal temperature for the microwave method was 100ºC, reaching a yield of 18.9
± 0.5 mg g⁻¹. The use of higher temperatures resulted in a dramatic decrease in the yield, as
compared to sealed vessels at the same temperatures. This suggests the occurrence of specific
microwave effects that result in the degradation of PCB, which was confirmed by the relative
low product purity. The product purity was found to be lower for the microwave assisted
method (Fig. 5B.), as compared to the sealed vessel method for the same reaction time and
despite the temperature being 20ºC lower. This shows that the so called specific microwave
effects also promote the release of a higher amount of reaction by-products.

3.2.2. Effect of the reaction time on the yield and purity

Tests at different reaction times for the sealed vessel and microwave methods showed
that 30 min was the time necessary to achieve the maximum yield, as shown in Fig. 4C.
Longer reaction times resulted in product degradation in both cases, although the effect was
more pronounced for the microwave assisted method probably due to the presence of microwave irradiation.

The reaction time also had an influence in the product purity. The conventional method, despite having a lower reaction temperature, which should result in a milder reaction, resulted in lower purity (38 ± 4 % w/w) than the sealed vessel method (62.2 ± 0.9 % w/w). This is attributed to the long reaction time, as the chances of the formation of by-products are higher in 16 hours of reaction compared to 30 min at higher temperatures.

3.2.3. Effect of the solvent-phycocyanin ratio on the yield

The effect of the solvent-phycocyanin ratio was investigated in order to determine whether the yield could be improved by using more solvent, or whether less amount of solvent could suffice to achieve the same yield. Three different solvent-phycocyanin ratios were tested for the sealed vessel cleavage method at 120 ºC for 30 min using ethanol 96% as reagent. The volume of solvent was maintained constant at 10 ml in order to avoid variations associated with the heat distribution in different amounts of solvent, whereas the amount of phycocyanin was adapted to fulfil the desired ratios.

The ratios tested were 50, 100 and 150 ml solvent per g of phycocyanin. Increasing the ratios from 100 to 150 ml g⁻¹ phycocyanin resulted in statistically identical yields: 20.7 ± 0.7 % w/w and 21.3 ± 0.4 % w/w, respectively. This suggests that using larger amounts of solvent resulted in the cleavage of just as much PCB, but more diluted. It is therefore concluded that the yield obtained at a ratio of 100 ml g⁻¹ is the maximum that can be achieved using this method. On the other hand, the ratio of 50 ml g⁻¹ provided a lower yield, probably due to losses during filtration of the highly concentrated solution. It is therefore concluded that the
ratio of 100 ml g$^{-1}$ is the optimal choice, as it reaches the maximum yield with moderate solvent consumption.

3.3. Reaction mechanism

The reaction mechanism is the same for the three cleavage methods. It is believed to be a solvolysis, in which the solvent (reagent) reacts with the substrate. In this case the reagents were neutral alcohols, which are weak bases and poor nucleophiles. However, in the absence of any other reagent, alcohols can undergo solvolysis reactions acting both as bases and nucleophiles, giving a mixture of elimination and substitution products. The reaction takes place by two competing pathways: a concerted E2 elimination and a $S_N2$ nucleophilic substitution. A stable intermediate carbocation is not expected, as the cysteine thiolate is not a good leaving group. Since the solvent reagent is always in large excess concentration, the reaction follows pseudo-first order kinetics (Malwade, Roda-Serrat, Christensen, Fretté, & Christensen, 2016). The reaction mechanisms are shown in Fig. 3. In the E2 elimination pathway, the reagent acts as a base and accepts the beta hydrogen to the carbon involved in the thioether bond, resulting in the formation of a double bond adjacent to ring A, and the concerted release of the cysteine thiolate. This reaction leads to the formation of the two PCB isomers (See section 3.1.1.). Bishop et al. suggested the reaction to take place via thiol elimination, however they refer as the assignment of mechanism as “tenuous” (Bishop, et al., 1986).

The competing pathway is a nucleophilic substitution ($S_N2$) taking place at the carbon involved in the thioether bond. The reagent attacks the thioether carbon and the cysteine thiolate is released in a concerted way, leading to the formation of the reagent-PCB adduct. This hypothesis was confirmed by the finding of specific solvent adducts for each of the two
different reagents tested: methanol and ethanol. In both reaction mechanisms, the reaction is assisted by solvation of the thiolate leaving group, which most likely accepts a proton to become a thiol.

3.3. Effect of the cleavage on the spectroscopic properties of PCB

In Fig. 1. the visible absorbance profile of Linabluue G1® and purified PCB are shown at pH 7, where it is shown how they have the same visible absorbance maximum at 620 nm. However, in acidic conditions the visible maximum of PCB is shifted to 660 nm, showing pH sensitivity similar as reported for phycocyanin (Crespi & Smith, 1970). Therefore an appropriate strategy for stabilization of PCB is still needed.

4. Conclusions

Conventional reflux in methanol is the most common technique for cleavage of PCB from phycocyanin. This method requires reaction times in the range of 16 hours to achieve equilibrium. The use of the sealed vessel method allowed the increase of temperature above the solvent boiling point, and resulted in a marked increase in the reaction rate. This observation is attributed to a pure thermal kinetic effect. However, the incorporation of microwave irradiation resulted in most cases in degradation of the products for the same temperatures tested in the sealed vessel method. This suggests the presence of microwave specific effects other than thermal effects. The optimal results for cleavage of PCB were observed for the sealed vessel method carried out at 120 ºC for 30 min, and using a solvent-phycocyanin ratio of 100 ml g⁻¹, providing a yield of 20.7 ± 0.7 mg g⁻¹, and a purity of 62.2 ± 0.9 % w/w. The cleavage of PCB from phycocyanins by solvolysis is believed to take place via two competing reaction pathways, an E2 and S_N2 reaction, which result in the formation
of (3E)- and (3Z)-PCB isomers in the elimination pathway, and reagent specific solvent adducts in the substitution pathway. The optimization of process conditions for PCB cleavage opens a door to fast production of PCB from phycocyanins. The process suggested could be performed in larger scale using a jacket stirred stainless steel reactor that could take the 4.4 bar that reach ethanol at 120°C (Poling, Prausnitz, & O'connell, 2001). The purchase cost of such a reactor has been estimated to be three times lower than the cost of a batch stainless steel reactor big enough to process the same amount of product per unit time according to the purchase price estimation methods of Peters et al. (2003). This can be the first step towards the development of a new blue colourant from natural origin, provided the stability issues of the molecule are improved by careful product design.

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References


http://www.ecfr.gov/cgi-bin/text-idx?SID=6ffd146f772f44d1b7b76af13be18518&node=21:1.0.1.1.27&rgn=div5#21:1.0.1.1.27.1.31.33 Accessed June 30, 2017.


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

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

Fig. 2. Analytical HPLC chromatograms of the product mixtures obtained with the four reagents tested by conventional reflux cleavage.

Fig. 3. Reaction mechanisms for cleavage of phycocyanobilin by solvolysis. In reactions with methanol R = CH₃, and with ethanol R = CH₂CH₃.

Fig. 4. Yield [mg g⁻¹] and purity [% w/w] for the different reagents used in conventional reflux solvolysis of phycocyanin.

Fig. 5. (A) Yields [mg g⁻¹] of solvolysis reactions with ethanol 96% v/v for different cleavage methods and temperatures; (B) Purity [% w/w] obtained using different reaction methods; (C) Yields [mg g⁻¹] of solvolysis reactions with ethanol 96% (v/v) for different reaction methods and reaction times.
**Table 1.** Experimental outline. Different cleavage methods, schematic representation and parameters used.

<table>
<thead>
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