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Separation and quantitation of phycobiliproteins using phytic acid in capillary electrophoresis with laser-induced fluorescence detection

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Abstract

The similar electrophoretic mobilities and sizes of several of the phycobiliproteins, which are derived from the photosynthetic apparatus of cyanobacteria and eukaryotic algae, render their separation and quantitation a challenging problem. However, we have developed a suitable capillary electrophoresis (CE) method that employs a phytic acid–boric acid buffer and laser-induced fluorescence (LIF) detection with a single 594 nm He–Ne laser. This method takes advantage of the remarkably high quantum yields of these naturally fluorescent proteins, which can be attributed to their linear tetrapyrrole chromophores covalently bound to cysteinyl residues. As such, limits of detection of $1.18 \cdot 10^{-14}$, $5.26 \cdot 10^{-15}$, and $2.38 \cdot 10^{-15}$ mol/l were obtained for R-phycoerythrin, C-phycocyanin, and allophycocyanin proteins, respectively, with a linear dynamic range of eight orders of magnitude in each case. Unlike previously published CE–LIF methods, this work describes the separation of all three major classes of phycobiliproteins in under 5 min. Very good recoveries, ranging from 93.2 to 105.5%, were obtained for a standard mixture of the phycobiliproteins, based on seven-point calibration curves for both peak height and peak area. It is believed that this development will prove useful for the determination of phycobiliprotein content in naturally occurring cyanobacteria populations, thus providing a useful tool for understanding biological and chemical oceanographic processes.

Keywords: Phycobiliproteins; Proteins; Phytic acid; Allophycocyanin; Phycoerythrin; Phycocyanin

1. Introduction

Phycobiliproteins are water-soluble, highly fluorescent proteins derived from cyanobacteria (blue–green algae) and red algae [1], in which they can constitute up to 60% of the soluble protein content [2]. They are typically classified on the basis of their color into larger groups, the most common being phycoerythrins (PEs), phycocyanins (PCs), and allophycocyanins (APCs). The phycoerythrins look pink in color, whereas the phycocyanins and allophycocyanins appear dark blue and blue–green, respectively. These groupings reflect variations among the proteins in the specific profile of their absorbance spectra and the exact location of their absorbance maxima. By absorbing energy in the 470–670 nm region, these proteins enable their organisms to extend the range of their spectral absorption to collect light that is not otherwise absorbed efficiently by chlorophyll *a* [2,3].

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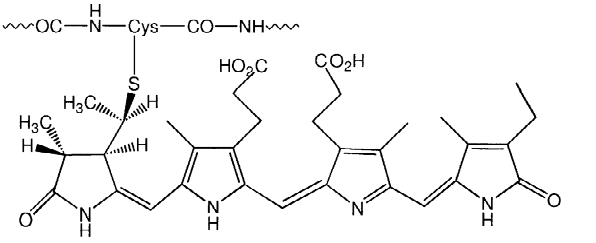
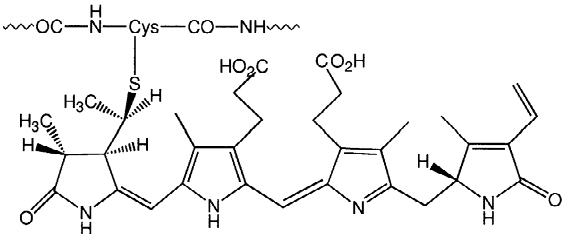
Phycobilisomes are subcellular structures of cyanobacteria, within which the phycobiliproteins are arranged in order to maximize the transfer of fluorescence resonance energy from PE to PC to APC, and finally on to the photosynthetic reaction center (chlorophyll *a*). This energy transfer chain is so effective that the phycobiliproteins in intact phycobilisomes are virtually non-fluorescent [4]. However, the phycobiliproteins become highly fluorescent in a region of the spectrum that is well separated from the autofluorescence of other biological cell matter when they are released from the subcellular phycobilisome structure.

All phycobiliproteins are oligomers of α- and β-subunits, although some also contain a γ-chain [4]. These subunits are composed of a protein backbone to which linear tetrapyrrole chromophoric groups are covalently bound by way of thioether linkages to cysteinyl residues [4], as seen in Fig. 1. The chromophores, also referred to as bilins or phycobilins, define the unique spectral characteristics of the phycobiliproteins, depending on the number and type of bilins present, the protein environment, and the steric relationship to one another (see Table 1). APC and PC primarily contain the phycocyanobilin chromophore, while PE contains primarily phycoerythrobilin, along with some other minor bilins (phycourobilin, cryptoviolin, and 697-nm bilin) [3]. The letter prefixes associated with the phycobiliprotein name (C-, R-, and B-) refer to historical nomenclature indicating the algal source [7].

The measurement of phycobiliproteins is not yet as routine or as accurate as the measurement of chlorophylls or carotenoids. Previous work in this area relied heavily on the use of fluorimetry, including that of Stewart and Farmer [8], who measured phycobiliproteins in freshwater and estuarine species; that of Wyman [9], who measured the PE content of *Synechococcus* strain WH7803; and of Vernet et al. [10], who used scanning spectral fluorescence measurements to estimate the PE concentration of extracted bulk samples. More recently, however, Campanella et al. [11] employed low- and high-performance liquid chromatographies to separate C-PC and APC, albeit these separations required 310 and 25 min, respectively. Similarly, Zolla and Bianchetti [12] developed a HPLC method (coupled with





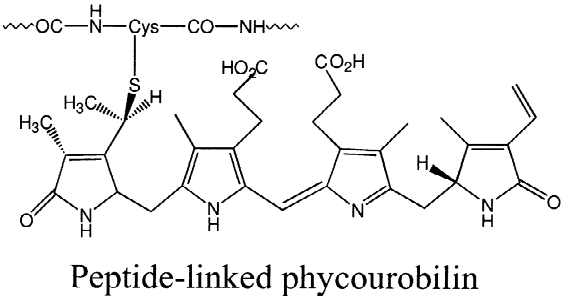


Fig. 1. Common phycobilins: linear, tetrapyrrole chromophoric groups, covalently bound to polypeptide chains of phycobiliproteins by way of thioether linkages to cysteinyl residues.

electrospray ionization mass spectrometry) for the determination of APC and PC in about 30 min, with two peaks evident per protein, which were attributed to the α- and β-subunits of each. Viskari et al. [13] were the first to report the use of capillary electrophoresis (CE) with laser-induced fluorescence (LIF) detection for the determination of phycobiliproteins. In that preliminary work, the separation of three phycobiliprotein groups was explored by using sodium dodecyl sulfate (SDS) and putrescine as buffer additives. Two of the three biliproteins were well separated in each buffer system in less than 5 min. However, in each buffer system, one of the proteins

Table 1
Physical and spectral properties of some common phycobiliproteins (compiled from Refs. [3,5,6])

Phycobiliprotein	Molecular mass	$\lambda_{max,\ ex}$ (nm)	$\lambda_{max,\ em}$ (nm)	Molar absorptivity ($cm^{-1}\ M^{-1}$)	Fluorescence quantum yield	Structure
APC	104 000	652	660	730,000	0.68	$(a,\beta)_3$
C-PC	232 000	620	647	1,540,000	0.51	$(a,\beta)_6$
R-PE	240 000	565 (480)	578	1,960,000	0.82	$(a,\beta)_6\gamma$
B-PE	240 000	565 (546)	575	2,410,000	0.98	$(a,\beta)_6\gamma$

could not be resolved from the other two or else the protein peaks were split, (not unlike the multiple peaks seen for APC and PC in Zolla and Bianchetti's HPLC work), thus resulting in overlap.

Recently, the use of the dodecasodium salt of inositol-hexaphosphoric acid, better known as phytic acid, has been reported to improve separation efficiency and resolution of proteins [14–17] and peptides [18,19]. Phytic acid is a naturally occurring nontoxic compound containing six hexane-ring bound phosphate groups with pK_a values ranging from 1.9 to 9.5 [20]. The sodium salt of phytic acid is highly soluble in aqueous media and it is UV transparent down to 200 nm. Phytic acid has a strong polyanionic character over a wide pH range and, therefore, it binds with the positively charged amino acid residues on the proteins and peptides, particularly with lysine and arginine [14,21]. This ion-pair formation enhances the efficiency and resolution of protein analysis in CE by reducing the net positive charge of basic proteins, therefore, resulting in reduced Coulombic interaction with the negatively charged silanol groups on the capillary surface. Both acidic proteins (such as phycobiliproteins with their pK_a values around 4.5) and phytic acid have a net negative charge at typical, near-neutral CE buffer conditions. However, they also form complexes and possible mechanisms include direct binding of phytic acid to protonated α-NH_2 terminal groups and ϵ-NH_2 groups of lysine residues, and a multivalent cation-mediated interaction [22].

In the present work, then, we describe the use of phytic acid as an additive to the electrophoresis buffer used to separate and quantify all three phycobiliproteins in a mixture. Such a complete separation was not possible in previous work employing putrescine- and SDS-containing buffers [13]. Also in the present work, we conveniently employed a single He–Ne laser for the simultaneous excitation and detection of all three phycobiliproteins following their separation by CE. Detection limits, limits of quantitation, and linear dynamic ranges for APC, PC and PE are also reported, as well as percentage recoveries for each protein from a synthetic mixture of the three.

2. Materials and methods

2.1. Reagents, buffers, and samples

All three phycobiliproteins, APC, C-PC and R-PE, were bought from ProZyme (San Leandro, CA, USA) at concentrations of 17.0 ($1.6\cdot10^{-4}$ mol/l), 21.0 ($9.1\cdot10^{-5}$ mol/l) and 20.0 ($8.3\cdot10^{-5}$ mol/l) mg/ml, respectively. They were supplied as suspensions in 60% ammonium sulfate, 50 m*M* sodium phosphate (pH 7.0), and 5 m*M* sodium azide. They were stored protected from light at 4 °C for up to 1 year. Stock solutions of these proteins were prepared by diluting the appropriate amounts of each protein in Milli-Q distilled, deionized water (Millipore, Bedford, MA, USA). Stock protein solutions prepared in this way were stored protected from light at 4 °C for no more than 1 day. For detection limit studies and construction of calibration curves, each protein's stock solution was serially diluted with Milli-Q water over the desired concentration range.

Buffers were prepared by dissolving boric acid at 50 m*M* concentration (J.T. Baker, Phillipsburg, NJ, USA) in Milli-Q distilled, deionized water and adjusting the pH to the desired value (8.15) by the addition of 0.1 and/or 1.0 *M* NaOH (Fisher Scientific, Fair Lawn, NJ, USA). The sodium salt of phytic acid (Sigma, St. Louis, MO, USA) was used as a buffer additive at 10 m*M* concentration. All buffers were stored in plastic bottles at room temperature, and were filtered through 0.20 μm nylon

syringe filters (Corning, Corning, NY, USA) prior to use.

2.2. Instrumentation

The experiments were carried out on a Bio-Rad BioFocus 3000 CE system with LIF detection (Hercules, CA, USA). Samples were pressure injected at 5 p.s.i. s in each case (1 p.s.i.=6894.76 Pa). Excitation was achieved with the 594 nm line of a yellow He–Ne laser (JDS Uniphase, Manteca, CA, USA) for all phycobiliproteins with emission selected by a filter (Omega Optical, Brattleboro, VT, USA) centered at 630 nm. Unless otherwise stated, 30 cm×50 μm I.D. uncoated fused-silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) were used. The effective length from inlet to detector was 25.4 cm. Every day before the first run, the capillary was conditioned by pressure flushing it with 0.1 *M* NaOH at 100 p.s.i. for at least 5 min, then it was allowed to sit with NaOH in the capillary for 10 min and flushed again with NaOH for about 2 min followed by a 1-min water flush and a 10-min buffer flush. Subsequently, and before each run, the capillary was pressure rinsed with the running buffer for 25 s at 100 p.s.i. Capillary, sample, and buffer temperatures were maintained at 20 °C throughout the experiments, unless otherwise noted.

2.3. Procedures

Limits of detection and linear ranges were determined by preparing a series of standard solutions from the protein stock solutions. Seven-point calibration curves were constructed using both peak heights and areas from the runs for the phycobiliprotein standards that were prepared from the protein stock solutions. The running conditions for all experiments were: 30 cm capillary, 10 kV voltage, 5 p.s.i. s injection, 20 °C, 50 m*M* borate–10 m*M* phytic acid (pH 8.15) buffer, detection at 594/630 nm.

3. Results and discussion

To improve phycobiliprotein separation efficiency and resolution, we used phytic acid as an additive to a 50 m*M* borate running buffer (pH 8.15). Phytic acid concentrations ranging from 5 to 20 m*M* were tested, and 10 m*M* was chosen as optimal, since it afforded the best separation between biliprotein peaks. In addition, analysis times were less than 5 min using this buffer with a 25.4 cm capillary (effective length) and a field strength of 333 V/cm. Higher concentrations of phytic acid did not improve the separation significantly but did result in increased migration times. Lower concentrations of phytic acid did not afford complete separation of the three proteins. Furthermore, the borate buffer pH was varied from 7.3 to 9.8, and 8.15 was chosen as optimal due to a combination of the best resolution, peak shape, and fluorescence intensity at this pH.

Fig. 2 shows electropherograms of the three phycobiliproteins run (a) independently and (b) together in the borate–phytic acid buffer system. As can be seen, the purified APC sample resulted in the appearance of two peaks with equal heights, centered around 4 min. The pure C-PC sample behaved similarly, with its first peak overlapping that of APC and its second peak appearing around 3.7 min. R-PE showed a single, broader and less intense peak at 4.5 min. The existence of multiple peaks for a single, purified biliprotein is a common phenomenon with different buffer additives. We previously reported similar results with SDS- and putrescine-containing running buffers [13]. Multiple peaks in the electropherograms for the single proteins APC and C-PC may be due to the formation of different phytic acid–protein complexes, although this would not be favored by electrostatics. More likely, the two peaks observed for each of APC and C-PC could be attributed to the separation of the different subunits (α and β) of the proteins, as concluded by Zola and Bianchetti [12], or alternatively, to the separation of the monomer and trimer or hexamer forms of APC and C-PC, respectively, since it is known that at low concentrations the proteins tend to dissociate from their aggregate states—especially APC [23]. It is also possible that the multiple peaks observed in the electropherograms for APC and for C-PC could be due to the separation of multiple forms of the proteins having different isoelectric points. However, isoforms are more commonly displayed by biliproteins from cryptomonad sources as opposed to biliproteins from cyanobacterial and red algae

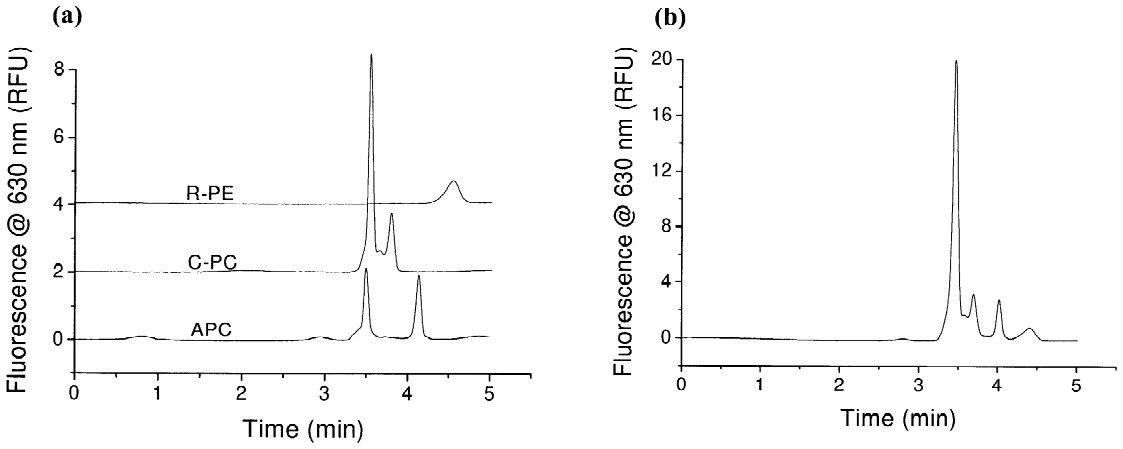


Fig. 2. Electropherograms recorded for (a) individual phycobiliproteins (0.85 μg/ml APC, 1.05 μg/ml C-PC, 4.00 μg/ml R-PE) and (b) a protein mixture (0.98 μg/ml APC, 3.65 μg/ml C-PC, 3.86 μg/ml R-PE). Separations employed a 50 m*M* borate buffer (pH 8.15) with 10 m*M* phytic acid, and an uncoated silica capillary [30.0 cm (25.4 cm effective length)×50 μm I.D.]. Other conditions: 5 p.s.i. s injection; 10 kV separation voltage; 20 °C.

sources [24], as employed in this study. Interestingly, only the R-PE appeared as a single, broad peak in this phytic acid-containing buffer system, whereas it presented itself as multiple peaks in the SDS–phosphate buffer system described previously [13].

The electropherogram of a mixture of APC (0.98 μg/ml), C-PC (3.65 μg/ml) and R-PE (3.86 μg/ml) can be seen in Fig. 2b. The first tall peak just before 3.5 min represents an overlap of the first peak attributable to each of APC and C-PC, while the remaining three peaks can be attributed to the individual proteins C-PC, APC and R-PE, respectively. The average resolution between the C-PC and APC peaks for two replicate runs (one of which is shown in Fig. 2b) was 2.20, whereas it was 1.91 between APC and R-PE. The average number of theoretical plates per meter for two replicate runs were 27 650, 75 992 and 14 528 for C-PC, APC and R-PE, respectively. Duplicate electropherograms were recorded for phycobiliprotein mixtures with concentrations ranging from 0.98–4.92, 3.65–18.24 and 3.86–19.30 μg/ml for APC, C-PC and R-PE, respectively, and the overall average resolution and number of plates are presented in Table 2. Values differed by no more than 10% from the reported averages, regardless of the concentration within the stated range. The number of theoretical plates was obtained from the Bio-Rad BioFocus integrator

Table 2
Figures of merit for APC, C-PC and R-PE

Phycobiliprotein	Number of theoretical plates/m*	Resolution*	Limit of detection, μg/ml (mol/l)	Limit of quantification, μg/ml (mol/l)	Upper limit of linear range (μg/ml)	Linear dynamic range
C-PC	25 035		$1.22\cdot10^{-6}$ ($5.26\cdot10^{-15}$)	0.10 ($4.31\cdot10^{-10}$)	75.0	8
		2.32				
APC	71 976		$2.47\cdot10^{-7}$ ($2.38\cdot10^{-15}$)	0.005 ($4.81\cdot10^{-11}$)	40.0	8
		1.88				
R-PE	14 000		$2.84\cdot10^{-6}$ ($1.18\cdot10^{-14}$)	0.20 ($8.33\cdot10^{-10}$)	175.0	8

*Column entries represent the average of 14 runs, ranging in concentrations from 0.98–4.92, 3.65–18.24, and 3.86–19.30 μg/ml for C-PC, APC, and R-PE, respectively.

software, which calculated them according to the formula:

$$N = 5.54 \cdot \left(\frac{t_r}{w_{1/2}} \right)^2$$

where N is number of plates, t_r is migration time, and $w_{1/2}$ is peak width at half height. The resolution between peaks was calculated manually according to the formula [25]:

$$R = 1.18 \cdot \frac{(t_{r,2} - t_{r,1})}{(w_{1/2,1} + w_{1/2,2})}$$

where R is resolution, and subscripts 1 and 2 refer to the first and second peaks in a neighboring pair.

Phycobiliproteins possess extremely high molar absorptivities and fluorescence quantum yields (see Table 1), making them ideal candidates for sensitive detection by laser induced fluorescence. Furthermore, the excitation and emission wavelengths for these proteins are at long enough wavelengths that interference from scattering and the autofluorescence of ubiquitous constituents of biological systems, such as porphyrins and flavins, is minimized [1]. To find the detection limits for our system, we prepared a set of standard mixtures of APC, C-PC and R-PE from stock solutions of each protein. Duplicate injections of each standard mixture were analyzed by CE–LIF, employing the conditions stated in Fig. 2. From the resulting electropherograms, we constructed a calibration curve for each protein based on either the height or area of its corresponding peak, and calculated the limits of detection (3σ) from the baseline noise and the slopes of the calibration curves (see Table 3). As summarized in Table 2, the limits of detection (LODs) ranged from $1.18 \cdot 10^{-14}$ mol/l for R-PE to $2.38 \cdot 10^{-15}$ mol/l for APC. R-PE had the highest detection limit, which was not surprising since the excitation for the protein mixture took place at 594 nm and the detection was centered at 630 nm. These conditions are better suited for the phycocyanins than for R-PE, since its absorbance spectrum shows reduced sensitivity at these wavelengths. Optimum excitation/emission couples for C-PC, APC, and R-PE, as stated in Table 1, are 620/647, 652/660, and 565/578 nm, respectively.

Limits of quantification (LOQs) were also determined. The LOQ refers to the lowest injected concentration of protein that yields a peak clearly visible above the baseline noise (at 3σ) in the electropherograms recorded under the prevailing conditions described herein. Experimentally determined LOQs, as summarized in Table 2, follow the same trend as the LODs, with greatest sensitivity being achieved for APC under these conditions.

The upper limit of the calibration range for each protein was found by determining where peak heights for samples of increasing concentration began to deviate from linearity. The ranges extended up to 40 μg/ml for APC, 75 μg/ml for C-PC and 175 μg/ml for R-PE, as presented in Table 2. Based on these upper limits and the previously stated LODs, the linear dynamic range (LDR) for all three biliproteins was found to be eight orders of magnitude. These unusually high LDRs can be attributed to the low detection limits obtained for each of the phycobiliproteins, as evident in Table 2.

To demonstrate the utility of our CE–LIF method for the separation and quantitation of phycobiliproteins, we determined the percentage recovery based on initial, known concentrations of the three phycobiliproteins in a synthetic sample mixture. This

Table 3
Calibration curve data for APC, C-PC and R-PE

Phycobiliprotein	Height		Area	
	Calibration equation*	R^2	Calibration equation**	R^2
APC	$H = 40\,252c - 10\,439$	0.9803	$A = 2\,027\,901c - 510\,030$	0.9812
C-PC	$H = 8148c - 1657$	0.9872	$A = 535\,376c - 196\,448$	0.9892
R-PE	$H = 3508c - 5548$	0.9754	$A = 419\,673c - 716\,193$	0.9744

* H represents peak height (relative fluorescence units as given by BioFocus integration software) and c represents protein concentration (μg/ml).
** A represents peak area (time × relative fluorescence units).

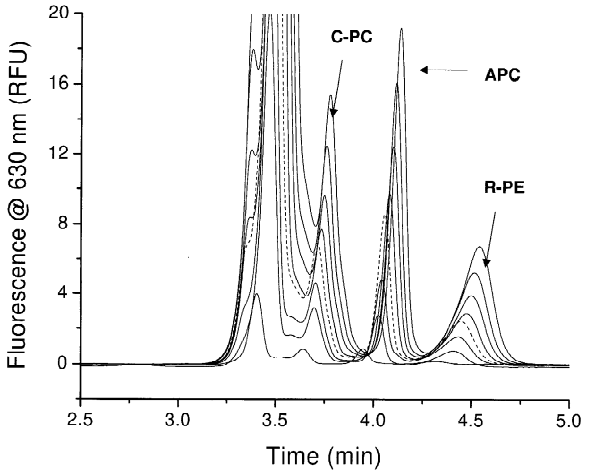


Fig. 3. Electropherograms recorded for seven standard mixtures (solid lines) and one synthetic sample mixture (dashed line) containing different concentrations of phycobiliproteins, ranging from 0.20 to 4.92 μg/ml for APC (2.17 μg/ml in sample mixture); 0.73 to 18.24 μg/ml for C-PC (8.02 μg/ml in sample mixture); and 0.77 to 19.30 μg/ml for R-PE (8.49 in sample mixture). All separation conditions as specified in Fig. 2.

synthetic mixture was prepared with known protein concentrations, selected to coincide mid-range within the concentrations for a seven-point calibration curve constructed for each of the three proteins. Electropherograms obtained for the seven standard protein mixtures along with the synthetic sample mixture are shown in Fig. 3. The concentration ranges for the proteins in the standard mixtures were 0.20–4.92, 0.73–18.24 and 0.77–19.30 μg/ml for APC, C-PC and R-PE, respectively. Two sets of calibration curves for each of the three biliproteins were prepared by plotting peak height and peak area versus concentration for each. Table 3 shows the data for the calibration curves, including the equations of the best-fit lines and their correlation coefficients (R^2 values). The average of two replicate runs was used to establish each of the seven points for each calibration line.

By substitution of the average peak height (or area) from duplicate runs corresponding to each of the three proteins in the synthetic sample mixture into the corresponding calibration equation, the resulting protein concentrations could be solved. These are presented in Table 4, where they are compared to the known concentrations used to prepare the synthetic mix. The resulting percentage recoveries are very good, ranging from 93.2 to 105.5%, depending on the protein. To assess the robustness of our method of phycobiliprotein separation and quantitation, we repeated this recovery study in its entirety five times over a period of 16 weeks. Average recoveries (based on peak heights) were 104.3±5.0% for APC, 108.2±15.2% for C-PC and 97.8±4.2% for R-PE. Virtually no difference in the recoveries resulted if peak areas were used instead of peak heights for calibration. However, since the leading edge of the C-PC peak used for quantitation is not completely resolved from the first coincidental peaks of APC and C-PC (see Fig. 3), it seems that the use of peak heights for quantitative analysis in this system is likely to be more reliable than the use of peak areas.

Thus, we have been able to demonstrate the simultaneous determination of APC, C-PC and R-PE in less than 5 min by CE–LIF using a simple borate–phytic acid buffer system with an uncoated silica capillary. The analysis of mixtures of phycobiliproteins extracted from real ocean water

Table 4
Recovered and actual concentrations and recovery percentages for APC, C-PC and R-PE in a synthetic sample mixture, based on peak heights and peak areas

Phycobiliprotein	Height		Area	
	Recovered/Actual concentration (μg/ml)	Recovery (%)	Recovered/Actual concentration (μg/ml)	Recovery (%)
APC	2.28/2.17	105.1	2.29/2.17	105.5
C-PC	8.35/8.02	104.1	8.30/8.02	103.5
R-PE	7.96/8.49	93.8	7.91/8.49	93.2

and cultured samples are ongoing in our laboratory, with the intention of developing an analysis system suitable for shipboard assays of these important proteins.

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