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Mild and Efficient Extraction of Fluorescent *Chlorophyll a* from Spinach Leaves for Application as the Sustainable Emitter in Light-Emitting Electrochemical Cells

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Natural pigments are sustainable compounds that can be employed as emitters, sensors and sensitisers in optoelectronics. The most abundant pigment, chlorophyll, offers advantages of easily available and plentiful feedstock, biodegradability and non-toxicity. However, strenuous extraction and separation limit its application on larger scale. In this work, a practically mild and scalable extraction and separation method for rapid isolation of *chlorophyll a* from spinach is presented. Three different stationary phases for column chromatography were evaluated, and a new solvent system was developed for the elution of *chlorophyll a* on a neutral alumina chromatography column. The purified product was obtained with a yield of 0.98 mg·g^{-1} with respect to the dry leaves. A first light-emitting electrochemical cell (LEC) based on *chlorophyll a* as the emitter is reported, using the extracted *chlorophyll a* as the guest compound dispersed in a blend-host matrix in a concentration of 2.5 or 5 mass%. The higher-chlorophyll-concentration LEC exhibits emission solely from the chlorophyll emitter, with the main emission peak located at 675 nm. The lower-chlorophyll-concentration LEC features two distinct emission bands, one in the red region that is originating from the chlorophyll guest and one in the blue region (main peak at 430 nm) that stems from the blend host. This combined red: blue emission can be attractive for, e.g., greenhouse applications, since it matches the action spectrum of plant photosynthesis.

Introduction

Research and technological development in general, and in organic electronics in particular, is increasingly focused on sustainability, with many efforts directed towards replacing fossil-based raw materials with biomass for the synthesis of organic semiconductors. The use of bio-based materials is attractive due to the renewable feedstock and the intrinsic biodegradability and biocompatibility, which enable greener production and lowered end-of-life environmental impact of the associated products.[1,2] Bio-sourced pigments are, for instance, a promising feedstock for the synthesis of the emitters for light-emitting devices, and reported examples include cytochrome c,[3] myoglobin[3] and hemin.[4]

Chlorophyll a is a particularly interesting natural pigment because of its abundancy, as virtually any green plant material can be used for its extraction. Several applications of chlorophyll and its derivatives have been reported in dye-sensitised solar cells,[5–7] photo-induced hydrogen generation,[8,9] sensors[10,11] and phototransistors.[12,13] However, only a few publications describe the utilization of chlorophyll[14] or extracted plant pigments[15] in organic light-emitting diodes (OLEDs).

Chlorophylls and bacteriochlorophylls are the most abundant group of pigments in nature, where they function as the crucial element in the photosynthetic processes of plants, algae and many groups of bacteria. Their chemical structures vary, but all chlorophylls possess a cyclic tetrapyrrole core, called porphyrin, chlorin or bacteriochlorin depending on its oxidation state[16] (Figure 1a–c). The most common *chlorophyll a*, occurring universally in photosynthetic organisms, comprises a chlorin ring with a magnesium ion at its centre and a phytyl side chain (Figure 1d).

In plant cells, chlorophyll is found in the chloroplasts, i.e., the organelles responsible for harvesting solar energy and conducting photochemical reactions. When photons reach a chloroplast, they are absorbed by the pigments bound to the proteins of the light-harvesting complexes. These pigments include *chlorophyll a*, *chlorophyll b* and carotenoids. They absorb light of different wavelengths, which enables photosynthetic organisms to harvest a significant portion of the solar spectrum.[17] The efficiency of the photosynthesis in utilising the absorbed energy is high, as exemplified by that only around 1–5% of the chlorophyll molecules undergo radiative relaxation.[18,19] The consequence is however that, in order to utilise chlorophyll as an efficient emitter, it is necessary to first extract and separate it from the plant cells.

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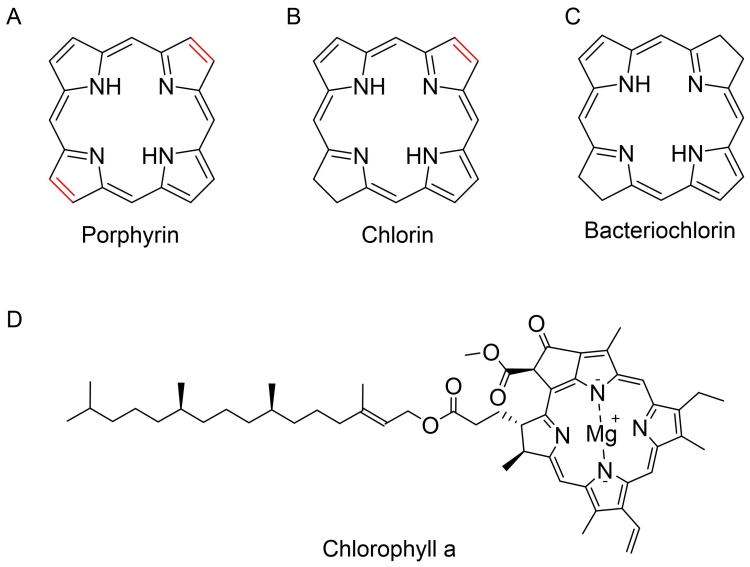

Figure 1. Chemical structures of porphyrin (a), chlorin (b), bacteriochlorin (c) and *chlorophyll a* (d).

Following the discovery and initial development of the OLED, further research in organic light emission led to the invention of the LEC.[20] Its fascinating (and complex) electrochemical doping operation[21] enables for an attractive low-cost printing fabrication of air-stabile and robust electrode/active-material/electrode device structures.[22] The LEC active material comprises an emitter blended with a solid electrolyte.[23] In contrast to the OLED, no separate carrier injection and transport layers are required because of the in-situ electrochemical doping operation.[24] To our knowledge, no research concerning the application of chlorophyll-based emitters in LECs has been published up to date.

In this work, *chlorophyll a* was isolated from spinach leaves. Spinach was selected due to its abundant availability and high chlorophyll content.[25] Two different methods for pigment extraction, blending and grinding, have been developed and evaluated with respect to scalable extraction. Three different stationary phases, in the form of silica gel, neutral alumina and powdered sucrose, were assessed for the column chromatography separation of *chlorophyll a.* Subsequently, a new eluent system was explored for the fast and large-scale pigment separation. The photoluminescence (PL) spectrum and the PL quantum yield (PLQY) for the derived neat *chlorophyll a* dissolved in ethanol solution was highly independent on the excitation wavelength and the PLQY was determined to be 29%. An LEC device equipped with air-stabile electrodes and featuring the isolated *chlorophyll a* as the emitter dispersed in an appropriate blend-host matrix delivered electroluminescence from the *chlorophyll a* emitter with a characteristic peak wavelength of 675 nm.

Results and Discussion

The investigation and development of chlorophyll as an optoelectronic material have been hindered by its limited commercial availability and high price. Small batches of 10 mg can currently be purchased at a high price of 1200 €. Therefore, it is motivated to develop low-cost methods for the efficient and scalable extraction and purification of larger amounts of chlorophyll. The extraction of chlorophyll from biological raw materials has been the topic of several publications, with a particular focus being on the breaking of the cellular structure and on the development of functional extraction media. The most common solvents are methanol, ethanol and acetone,[26–30] but other solvents, such as dimethyl sulfoxide[31,32] and dimethylformamide,[33] have also been investigated. More complex extraction media have been reported, such as supercritical CO_2,[34–36] aqueous solutions of surface-active ionic liquids[37] and aqueous solutions of non-ionic surfactants.[38] Enzyme-assisted extraction of chlorophyll derivatives[39] has also been studied. To facilitate the penetration of the cellular structure by the extraction medium, the biological source material is usually homogenised by grinding. However, other methods, such as sonication have also been employed.[40]

The larger-scale extraction of chlorophyll is rendered difficult by its tendency to degrade (Figure 2 Degradation pathways of chlorophyll a). One common degradation pathway, driven by acidic conditions and high temperature,[41] constitutes the demetallation of the aromatic core, leading to the formation of pheophytin. Chlorophyllide can also form during the extraction through the hydrolysis of the phytyl side chain. The degradation of chlorophyll in plants occurs due to the presence of chlorophyllase enzyme, which remains active even in high concentrations of organic solvents. Although the chlorophyllase enzyme can be deactivated by boiling, it comes at the expense of pheophytin formation.[33] Further degradation can occur through both enzymatic and non-enzymatic processes,[42] and the overall extraction process should therefore preferably be rapid.

In order to evaluate the efficiency of the different chlorophyll extraction methods, the amount of pigments

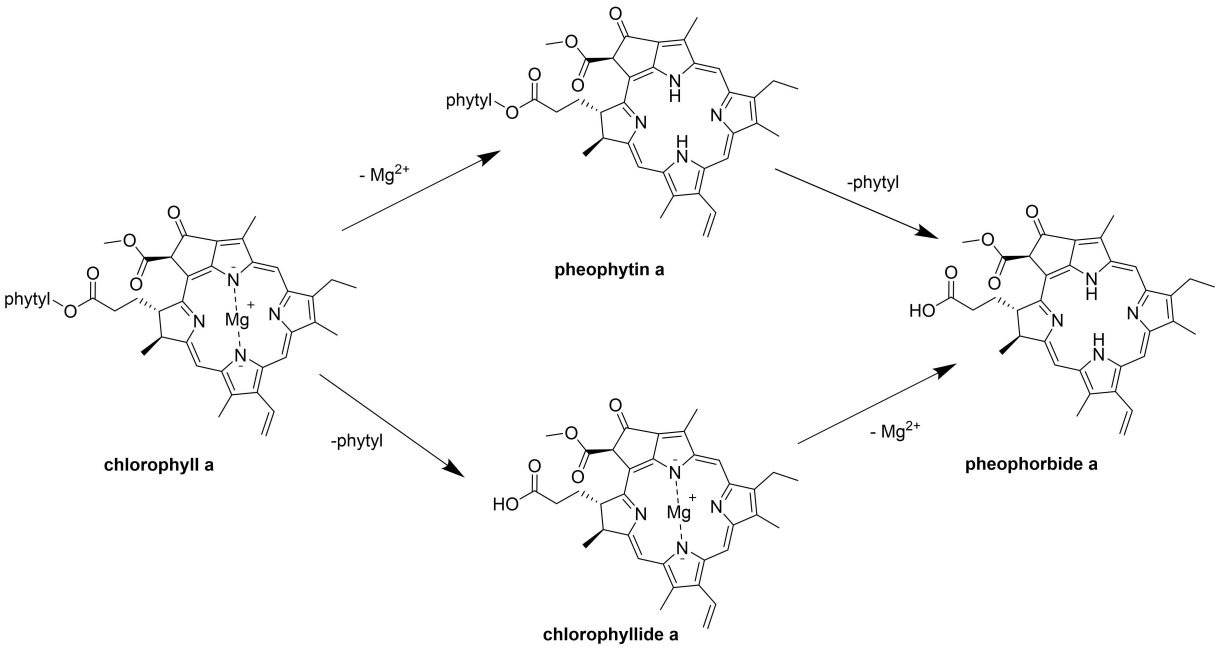

Figure 2. Degradation pathways of *chlorophyll a.* Acidic environment and high temperature lead to the demetallation of the aromatic core and the formation of *pheophytin a.* In the presence of water and chlorophyllase enzyme, the hydrolysis of the phytyl chain occurs, resulting in the formation of *chlorophyllide a.* The reported extraction and separation in mild conditions minimise the extent of degradation.

present in the source material needs to be measured. The determination of chlorophyll content in tissues of living organisms is fortunately common in biological research. In agriculture, chlorophyll measurements can provide a metric of environmental stress,[43] such as temperature,[44] drought,[45] light conditions,[46] acid rain[47] or pollution.[48] Chlorophyll content can also be correlated with crop yield to evaluate efficiency of farming methods[49] or used in physiological monitoring of plant ageing[50] and infections.[51] It has also been employed in taxonomical research[52–54] and ecological studies on pollution,[55] water eutrophication[56,57] and biodiversity.[58,59] Given this importance, it is not surprising that numerous spectrophotometric methods have been derived for measuring the chlorophyll content in both plant extracts[60–63] and in vivo.[64,65] The spectra of extracted pigment mixtures can be resolved with polynomial equations by measuring absorption at 2–4 different wavelengths in a specific solvent. Algorithms for methanol, ethanol and acetone solvents have mostly been reported, although acetone provides much sharper absorptions peaks of chlorophylls. However, acetone is an inefficient extractant of chlorophylls from some groups of plants, which leads to falsely low results of chlorophyll assays.[61,60]

Here, the content of chlorophyll in the spinach leaves for the extraction was determined spectroscopically. The methanol solvent was neutralised with a trace amount of magnesium carbonate to ensure acid-free conditions. The suspension was filtered to obtain a neutralised extraction solvent. 1.18 g of leaves were ground in a mortar with neutralised methanol until a homogenous, fine slurry was formed. The slurry was carefully transferred to a centrifuge tube, using neutralised methanol to wash down all the material, and refrigerated in the dark for 40 min. This sample was centrifuged for 20 min at 9000 rpm. The supernatant was decanted into a graduated cylinder, and the volume brought up to 50 ml. Solid pellets were discarded. The UV-Vis absorption spectrum was measured on a sample prepared by transferring 300 µl of the solution into a quartz cuvette and increasing the total volume to 2 ml by addition of neutralised methanol. The concentration of the chlorophyll was calculated with the following equation:[61]

$$[Chl\ a] = -2.0780A_{632} - 6.5079A_{652} + 16.2127A_{665} - 2.1372A_{696}\ (g \cdot m^{-3}).$$

and the extracted amount of chlorophyll with respect to the neat spinach leaves was found to be 1.28 $mg \cdot g^{-1}$.

The extraction process has been frequently reported, but it is notable that the subsequent isolation of the chlorophyll has rarely been discussed.[34,28,33,35,37] This stems from the fact that for most biological studies, the chlorophyll content can be determined spectroscopically without isolation. However, the applications of compounds in organic electronics often require that they are of high purity, which raises the need for an efficient separation of the chlorophyll from the other plant pigments. Separation procedures found in the literature are mostly based on high-performance liquid chromatography (HPLC)[66,67] and thin-layer chromatography (TLC),[68,69] which are designed for analytical rather than for preparative purposes. Column chromatography can be used for larger samples, using solvent systems developed for TLC, with several eluent compositions having been reported.[68] The employed stationary phases include silica gel, alumina and powdered sucrose. However, there is a lack of methods showcasing large-scale

isolation of chlorophyll from plant materials, although we note one such method that reports a chlorophyll yield of 0.24 $mg \cdot g^{-1}$ from dry spinach leaves.[70] Multistep purification procedures have also been investigated. One example combined a DEAE-Sepharose CL-6B ion exchange column with a Sepharose CL-6B column to separate *chlorophyll* a from *chlorophyll b*, with the yield being relatively high (0.44 $mg \cdot g^{-1}$ and 0.12 $mg \cdot g^{-1}$ respectively). However, the drawbacks were that the process was time-consuming (1 h elution time on the second column with 10 cm long bed for a 50 g batch) and that it required a pre-purification of the spinach extract by precipitation of chlorophyll complex with toxic dioxane in water.[71]

In this work, the pigments were extracted from spinach (*Spinacia oleracea*) leaves, purchased at a local supermarket in Gothenburg, Sweden, by two methods: grinding and blending. In the grinding method, the fresh spinach leaves were mixed with anhydrous magnesium sulfate and sand in 1:1:1 weight ratio, and the mixture was ground in a mortar until a fine powder was obtained. This powder was mixed with acetone in a 1:2 volume ratio in a bottle. The mixture was shaken, and thereafter left at rest for 30 min in order to allow the acetone to diffuse into the plant cells. The acetone extract was finally decanted through a filter and dried in vacuo.

In the blending method, the fresh spinach leaves were blended with a small amount of water in a kitchen blender for the formation of a fine slurry. The slurry was filtered on a Büchner funnel, and the separated water discarded. The solid filtrate was dispersed into acetone, and the plant fibres were removed by centrifugation. The pigments were finally extracted from the supernatant with ethyl acetate and dried in vacuo.

Both extraction methods were performed at ambient temperature to suppress heat-activated degradation processes (see Figure 2 and related discussion). Both methods resulted in satisfactory pigment extraction yield. The advantage of the grinding method was that the water present in the spinach leaves was absorbed by the anhydrous magnesium sulfate, and that no extraction in an organic solvent was needed. However, the blending method was much faster to execute and resulted in the extraction of larger amounts of pigments, which is important in order to inhibit degradation and enable cost-efficient scale up.

Column chromatography was used to separate the extracted pigments. Three different stationary phases were investigated: silica, neutral alumina and powdered sucrose (containing 2 mass% starch). The selected eluent for the initial trials was 70:30 (v/v) pentane:acetone, which resulted in sufficient solvent separation on silica TLC (Figure 3, left). The silica gel column provided sufficient separation between the bands (Figure 3, middle). However, an additional fraction appeared compared to TLC, which was identified as *pheophytin a*. The likely reason for its formation is the demetallation of *chlorophyll a* due to the acidic character of the silica gel. To avoid this degradation, neutral alumina was used as an alternative stationary phase. The eluent removed carotene very quickly, but the other pigments were strongly adsorbed on the alumina, forming very long bands that ultimately stopped

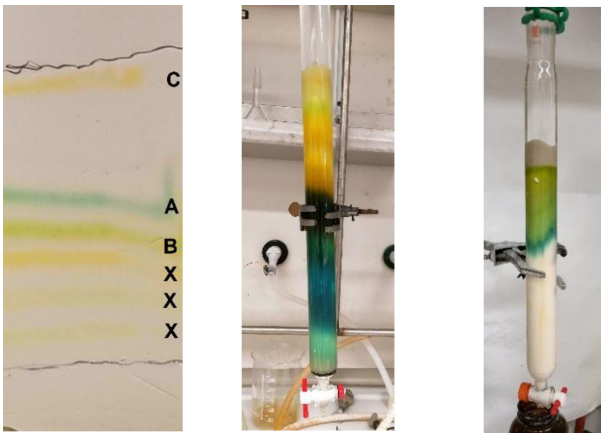

Figure 3. *Left:* Silica TLC of spinach extract using 70:30 pentane:acetone as eluent. Visible fractions are carotene (C), *chlorophyll a* (A), *chlorophyll b* (B) and xanthophylls (X). *Middle:* Separation of the extracted pigments on silica gel chromatography column. *Right:* Elution of *chlorophyll a* fraction on alumina chromatography column.

moving down the column with the eluent. The powdered sugar column did not perform well, as it clogged quickly, probably due to that the powder absorbs water present in small amounts in the eluent, and the flow of the mobile phase thus stopped completely. Based on these observations, the alumina stationary phase was selected for further investigation, as it minimized the formation of degradation products.

In order to further improve upon the separation procedure, a new solvent system was developed for the alumina column. At first, 90:10 (v/v) pentane:acetone was used to elute carotene and some xanthophylls, while other pigments remained on top of the column. Then, 99:1 (v/v) pentane:methanol was used to elute *chlorophyll a* as the first fraction (Figure 3, right). Due to the selectivity of the two solvent systems, column chromatography separation could be accelerated by applying a moderate pressure. With this separation method, 0.16 $mg \cdot g^{-1}$ of *chlorophyll a* per mass of fresh leaves (0.98 $mg \cdot g^{-1}$ per mass of dry leaves) was isolated from the spinach. This means that 12.5 mass% of the initial chlorophyll in the leaves was successfully isolated. The purity of the final *chlorophyll a* was examined by thin layer chromatography and NMR spectrum (Figure S2), which showed that the final product is essentially free from the presence of other compounds.

UV-Vis absorption spectroscopy was used to characterise the obtained *chlorophyll a* in acetone solution and as a neat film (Figure 4). Two absorption peaks at 430 and 662 nm were observed for the *chlorophyll a* in acetone solution, which is in agreement with the literature.[72] The absorption spectrum of the neat *chlorophyll a* film exhibits a red shift of the absorption onset, and a large broadening of the absorption peaks, compared to the solution spectrum, with the absorption peaks located at 443 and 679 nm. This is attributed to the aggregation of the *chlorophyll a* molecules in the solid state.[73] The photoluminescence (PL) spectra and the PLQY were collected on a dilute *chlorophyll a* solution in ethanol at different excitation wavelengths (Figure 5). The absolute PLQY were measured with an integrating sphere connected to a spectrometer (C9920,

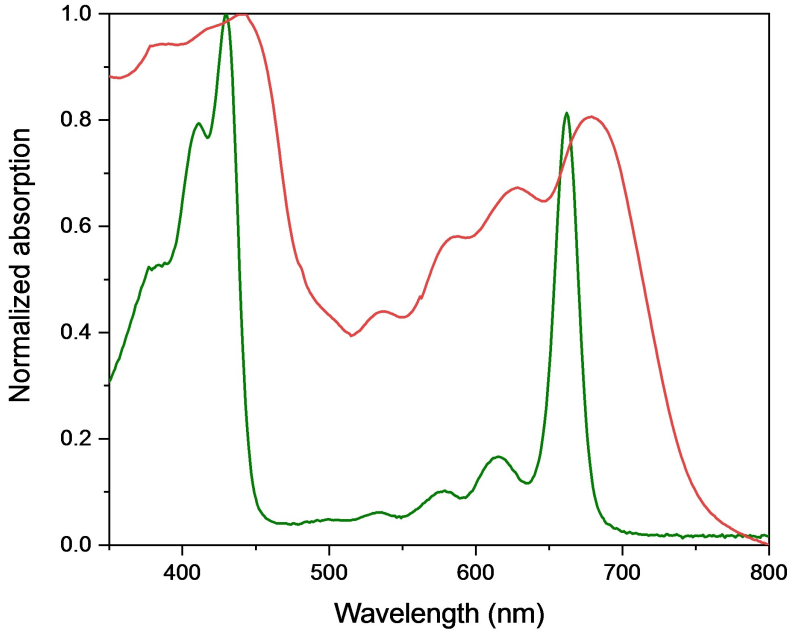

Figure 4. The normalised UV-Vis absorption spectrum of *chlorophyll a* in acetone solution (green) and as a thin film (red).

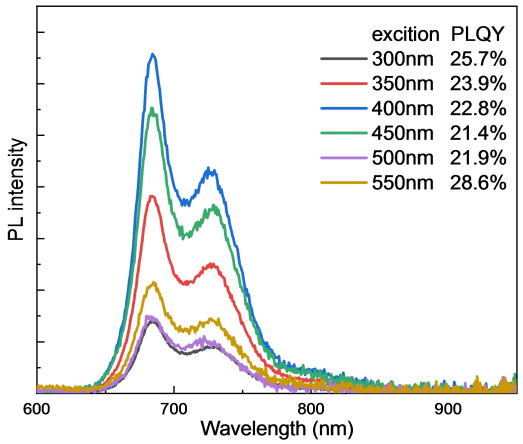

Figure 5. The photoluminescence spectra and the photoluminescence quantum yield of *chlorophyll a* in dilute ethanol solution at different excitation wavelengths. The independence of PL characteristics from the excitation wavelength demonstrates high purity of the product, as no other chromophores are present.

Hamamatsu Photonics). It is notable that both the PL spectrum and the PLQY were essentially invariant on the excitation wavelength, which imply that the extraction and isolation process has been successful in obtaining high-purity *chlorophyll a* and that this fluorophore emits from a single excited state. The highest value for the PLQY of 28.6% was recorded at excitation with a wavelength of 550 nm. No PL could be detected from the neat chlorophyll film, which implies strong aggregation induced quenching of the emission of neat chlorophyll in the solid state.[73]

Cyclic voltammetry was measured on a dilute solution of *chlorophyll a* in 0.01 M tetrabutylammonium hexafluorophosphate in acetonitrile (Figure S1), two Pt wires were the working and counter electrodes, and an Ag wire was the pseudoreference electrode. The latter was calibrated by the addition of a small amount of ferrocene at the end of the CV measurement, and the potential are reported with respect to the Fc/Fc^{+} redox couple. The derived onset potentials for the electrochemical oxidation (E_{ox}) and reduction (E_{red}) reactions are marked by the vertical dashed lines. These onset potentials were used for the determination of the HOMO and LUMO energy levels with the aid of the equations $HOMO = -(E_{ox} + 5.13)\ eV$ and $LUMO = -(E_{red} + 5.13)\ eV$. We find that the HOMO and LUMO values of the derived *chlorophyll a* are −5.27 eV and −3.73 eV, and that its electrochemical energy gap accordingly is 1.54 eV.

The issue with chlorophyll aggregation was addressed by the dispersion of the chlorophyll emitter into a PVK:OXD-7 blend-host matrix. The resulting films show a smooth surface as illustrated by the atomic force microscopy (AFM) images (Figure S4). The LEC devices were fabricated in an ITO/PEDOT:PSS/PVK:OXD-7:chlorophyll:$THABF_4$/Al architecture, with the chlorophyll guest concentration being either 2.5% or 5 mass%. The LEC devices were driven by a constant current density of 77 $mA \cdot cm^{-2}$. Figure 6a presents electroluminescence (EL) spectra recorded at steady state, whereas Figure 6b is a photograph of the uniform red emission from the high-chlorophyll-concentration LEC. Figure 6a shows that the major EL peak is located at 675 nm. A comparison with the PL spectrum of isolated or dispersed chlorophyll molecules (Figure 5) and the PL spectrum of a thin film of the majority PVK:OXD-7 blend host (Figure S3) reveals that this emission originates from isolated or dispersed chlorophyll molecules. We further find that the low-chlorophyll-concentration LEC features a second EL emission band, with its peak at 430 nm, which is assigned to residual blend-host emission.[74] We note that this combined red:blue emission might be interesting for greenhouse applications, since it

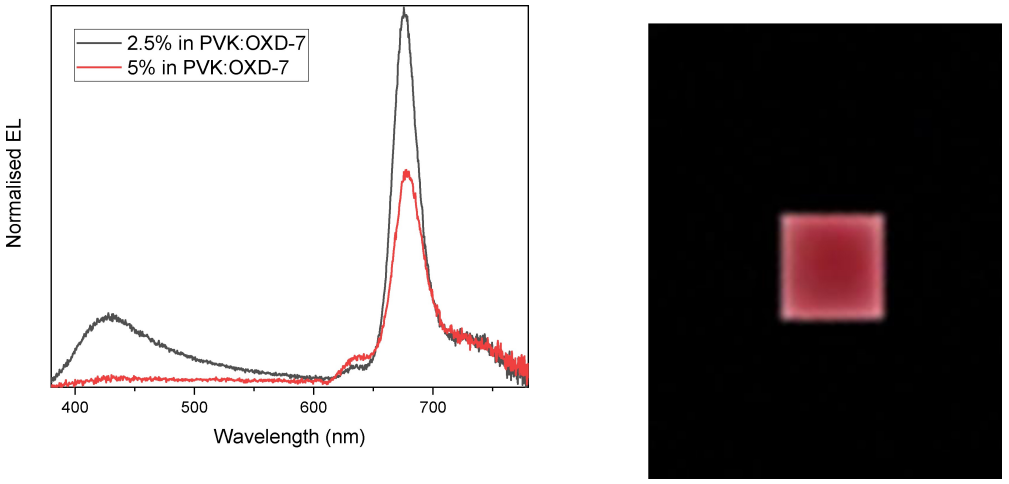

Figure 6. (a) The electroluminescence spectrum of ITO/PEDOT:PSS/PVK:OXD-7:chlorophyll:THABF$_4$/Al LEC devices, comprising a chlorophyll guest concentration of 2.5 mass% and 5% mass%, as specified in the inset. The devices were driven by 77 mA/cm^2 and the spectra recorded at steady state. (b) A photograph of the uniform red emission from the 5% chlorophyll-concentration LEC.

matches the action spectrum of plant photosynthesis. In the context of sustainability, we finally call attention to that the active material in this first chlorophyll-bioemiter LEC was fabricated by spin-coating from an environmental benign solvent in the form of anisole.[75]

Conclusions

A method for the mild and efficient extraction and separation of fluorescent *chlorophyll a* pigments from spinach leaves is presented. The spinach leaves were grinded and blended with acetone, and neutral alumina was determined to be a functional stationary phase for chromatographic separation in combination with a 99:1 (v/v) pentane:methanol eluent system. The overall process yield was 12.5%, but the main merit of the method lies in the abundancy and low cost of the raw material, and the rapid and selective separation of the *chlorophyll a* pigments. The practical functionality of the derived *chlorophyll a* pigments was demonstrated by their application as the emitter in LEC devices, which delivered unform red electroluminescence with the main emission peak at 675 nm. By tuning the active-material composition of these chlorophyll-LEC, a combination of blue and red emission was obtained, which matches the action spectrum of the photosynthesis process and thus could be of interest for the light source in industrial greenhouses.

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Conflict of Interests

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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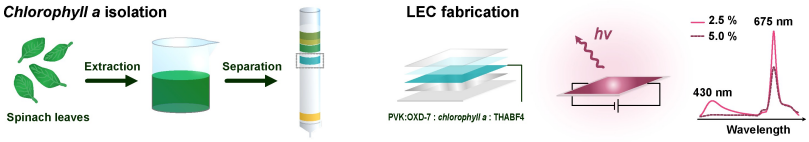

A rapid and scalable extraction and isolation method of chlorophyll a from spinach was developed. The isolated pigment was used to fabricate a light-emitting electrochemical cell (LEC) based on chlorophyll a as the guest-emitter dispersed in a blend-host matrix in a concentration of 2.5 or 5 mass%. The 2.5-mass% LEC exhibited combined red:blue emission attractive for greenhouse lighting applications.

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Mild and Efficient Extraction of Fluorescent *Chlorophyll a* from Spinach Leaves for Application as the Sustainable Emitter in Light-Emitting Electrochemical Cells