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An in-tube SPME device for the selective determination of chlorophyll *a* in aquatic systems

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ABSTRACT

We report a new device for the estimation of the content of chlorophyll *a* pigment in water samples as an indicator of water quality. The extraction of the pigment from water was also optimized. 10 mL of water was filtered through a nylon filter (45 μm pore size and 13 mm of diameter), after the chlorophylls were dissolved by immersing the filter in 1 mL of a low non-hazardous solvent as ethanol. An in-valve in-tube SPME device coupled to capillary liquid chromatography with diode array detection was designed. A capillary column of 70 cm in length (0.32 mm i.d. coated with 5% diphenyl–95% polydimethylsiloxane, 3 μm coating thickness) was used as the loop of the injection valve for preconcentration and a Zorbax SB C_{18} (SiO_2-based) 150 mm × 0.5 mm i.d., 5 μm column (Agilent) was used as analytical column. The achieved detection limit was 0.05 μg L^{-1} and the working range of concentrations was 0.1–1 μg L^{-1}. % RSD values between 2 and 11 were obtained. Chlorophyll *a* in several water matrices was determined with good results in presence of other pigments such as chlorophyll *b*, pheophytin *a* and pheophytin *b*.

1. Introduction

There are several types of chlorophylls (*a*–*d*) with slight differences in their molecular structure and constituents. Chlorophyll *a* (Chl *a*) is the main photosynthetic pigment and it is common to all phytoplankton. Chl *a* concentration is an indicator of phytoplankton abundance and biomass in waters [1,2]. Chl *a* can be used as an effective measure of trophic status, it is a potential indicator of maximum photosynthetic rate and it is commonly used as a measure of water quality. High levels often indicate poor water quality and low levels often suggest good conditions. The real problem is the long-term persistence of elevated levels [3]. Studies of the content of Chl *a* in the Spanish coast revealed the presence of this pigment at concentrations that varies between 1.6 and 0.33 μg L^{-1} depending on the years, season and months [4].

The most used analytical techniques for this determination are HPLC based methods as they allow the identification and quantification of chlorophyll pigments in several matrices such as functional drinks [5], teas [6], spinach leaves [7], olive oil [8] and waters [9,10]. Both underestimation and overestimation of chlorophylls by spectroscopic techniques have been described [11]. Additionally to separation techniques, extraction and preconcentration of chlorophylls are the other concern in their determination owing to the concentration levels in waters. By way of example, van Leeuwe et al. [12] published an extraction procedure based on the freeze-drying and extraction in acetone, the main drawbacks are excessive solvent consumption, a high volume of water sample and high time consumption.

Therefore, all these analysis requirements make the new in-tube microextraction preconcentration techniques (IT-SPME) coupled with capillary chromatographic systems especially attractive [13]. The use of these techniques not only improves the extraction efficiencies and sensitivity but also allows the automation of the whole analytical process [14,15]. Besides, an additional increment on the sensitivity can be reached substituting the conventional LC systems by capillary LC systems [16], providing a high speed analysis with low solvent consumption and waste generation [17,18].

The objective of this paper was to design a cost-effective analytical method for selective estimation of Chl *a* improving the characteristics of the reported methods. Miniaturization is the option used. For this purpose, we optimized an extraction procedure for this pigment from water samples, minimizing the use of hazardous solvents (only 1 mL of ethanol is employed) and we use IT-SPME preconcentration technique combined with capillary LC system using ethanol as organic solvent of the mobile phase. The proposed procedure improves sensitivity and analysis time with low organic solvent consumption and waste generation with respect to published methods.

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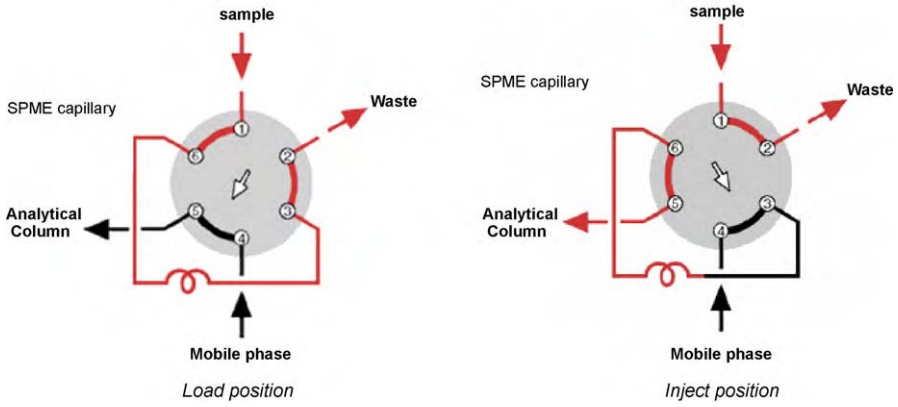

Fig. 1. Schematic diagram of the flow direction IT-SPME Capillary LC as function of the position of the injection valves.

2. Experimental

2.1. Reagents

Chlorophyll *a* and chlorophyll *b* (Chl *b*) were purchased from Sigma (St. Louis, MO, USA) and HPLC grade ethanol from Fluka (Buchs, Switzerland). The stock standard solutions of Chl *a* and Chl *b* were prepared by dissolving the appropriate amount of the pure solid in ethanol to obtain final concentrations of 45 and 144 mg L^{-1}, respectively. These solutions were stored in the darkness at 4 °C to avoid the formation of degradation products. Working standard solutions up to 10 μg L^{-1} were prepared daily by dilution in ethanol.

The standard solutions of pheophytin *a* and pheophytin *b* were obtained by adding 0.15 mL of 0.1 M HCl to 5 mL of a standard solution of Chl *a* and Chl *b* (1 mg L^{-1}), respectively.

Empore high performance C18 Extraction Disks (47 mm) and Bond Elut C18 200 mg extraction cartridges were from Varian (Haber City, USA). Nylon membrane filters 0.45 μm (13 mm) were obtained from Osmonics (Minnesota, USA).

2.2. Apparatus

The capillary LC system consisted in a capillary pump (Agilent 1100 Series, Waldbronn, Germany) equipped with a high-pressure six-port injection valve (Rheodyne model 7725), and a UV–vis diode array detector (Hewlett-Packard, 1100 Series). The absorbance signals for Chl *a* was registered at 430 nm for obtaining chromatograms. The detector was coupled to a data system (Agilent, HPLC ChemStation) for data acquisition and calculation. Spectra between 400 and 700 nm were registered for identification purposes.

2.3. Columns, mobile phases and chromatographic conditions

The analytical column employed was a Zorbax SB C_{18} (15 cm × 0.5 mm, 5 μm particle size) analytical column (Agilent, Waldbronn, Germany). A GC TRB-5 capillary column of 70 cm in length, and 0.32 mm i.d. coated with 5% diphenyl–95% polydimethylsiloxane (PDMS) (3 μm coating thickness) was used for the IT-SPME (Teknokroma, Barcelona, Spain).

Chromatographic separation was carried out in isocratic elution mode with a mixture of ethanol–water (95:5, v/v) at 20 μL min^{-1}.

2.4. Extraction procedures

Standard solutions and spiked water samples with chlorophyl *a* (injected concentration between 0.5 and 4 μg L^{-1}) were employed. Sample volume/elution volume ($V_{sample}/V_{elution}$) ratios between 10 and 40 were assayed. In all cases the experiments were carried out in the darkness to avoid photoxidation effects [19]. Three different extraction procedures were assayed. In the first procedure, extraction with nylon membrane disks, 10 mL of water sample was filtered through 0.45 μm nylon membranes (diameter 13 mm). The filters were immersed into ethanol (1, 0.5 and 0.25 mL), gently shacked. The second procedure, C18 cartridge extraction, was carried out using C18 SPE cartridges. In this case, 10 mL of water sample was passed through the cartridges where the analytes were retained. Chl *a* was eluted with 1, 0.5 or 0.25 mL of ethanol. Finally, the third procedure was based on the use of C18 extraction disk, 10 mL of water samples were passed through the disks and after those disks were immersed into 0.25 mL of ethanol. The ethanolic solutions were immediately measured.

2.5. In-tube solid phase microextraction

A CG open tubular capillary column TRB-5 of 70 cm was used as extracting phase and as injection loop. The capillary was placed in the high-pressure six-port valve replacing the injection loop. Capillary connections were facilitated by the use of a 2.5 cm sleeve of 1/16 in. polyether ether ketone (PEEK) tubing at each end of the capillary. 1/16 in. nuts and ferrules were used to complete the connections. Fig. 1 shows the schematic diagram of the direction flow for the IT-SPME preconcentration device and the connection with the analytical column.

In the load position of the injection valve 50 μL of standards or samples solution were manually passed through the capillary column. After the valve was rotated to the injection position and the desorption of Chl *a* was realized in the dynamic mode by flowing the mobile phase through the system.

2.6. Analysis of water samples

Seawater samples were collected at different sampling points in Valencia (Comunidad Valenciana, Spain) separated one from the other 40 km. The wastewater sample was collected from a treatment plant. The collection of samples was done in plastic bottles (1 L) and the samples were stored at 4 °C before their analysis.

Water samples were directly analysed with IT-SPME Capillary LC. Several replicates were made.

Table 1
Results obtained for the three extraction procedures assayed for Chl *a* in spiked water samples.

$V_{sample}/V_{elution}$	[a]Added Chl *a* ($\mu g\,L^{-1}$); $n=3$	[*]Found Chl *a* ($\mu g\,L^{-1}$); $n=3$ extraction method		
		Nylon membrane disk	C18 cartridge	C18 disk
10 mL/0.25 mL	0.5	n.a.	n.a.	0.5 ± 0.1
	1.0	n.a.	n.a.	0.6 ± 0.1
	2.0	1.7 ± 0.1	0.5 ± 0.1	n.a.
	4.0	n.a.	0.6 ± 0.1	n.a.
10 mL/0.5 mL	1.0	1.1 ± 0.1	1.3 ± 0.1	n.a.
	2.0	1.7 ± 0.1	1.5 ± 0.1	n.a.
10 mL/1 mL	1.0	1.1 ± 0.1	n.a.	n.a.
	2.0	1.8 ± 0.1	2.2 ± 0.2	n.a.
	2.9	2.7 ± 0.2	2.1 ± 0.2	n.a.

[a] Injected concentration; n.a. not assayed.

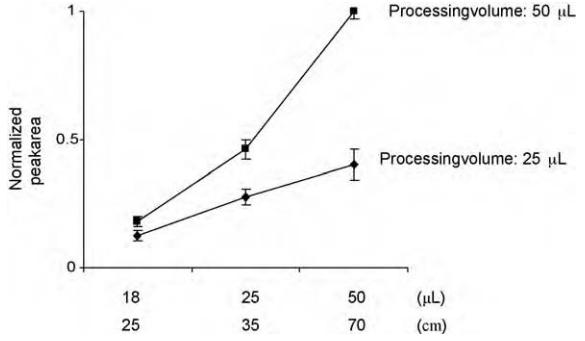

Fig. 2. Normalized chromatographic areas obtained for Chl *a* standard solution (injected concentration 5 $\mu g\,L^{-1}$) as function of the loop size.

3. Results and discussion

3.1. Study of the extraction procedure

Three extraction procedures (Section 2.3): extraction with nylon membrane disks, C18 cartridges extraction and C18 disks extraction were evaluated as function of the recoveries obtained for spiked water samples with Chl *a*. The results obtained are shown in Table 1. The chemical interactions that picoplankton (0.2–2.0 μm) established with the C18 cartridges or disks hindered the Chl *a* elution with ethanol providing low extraction efficiencies. The results obtained revealed that extraction with nylon membrane filter procedure using a preconcentration factor of 10 (V_{sample} 10 mL/$V_{elution}$ 1 mL) allowed the quantitative extraction of Chl *a* from water samples and were independent of the concentration level.

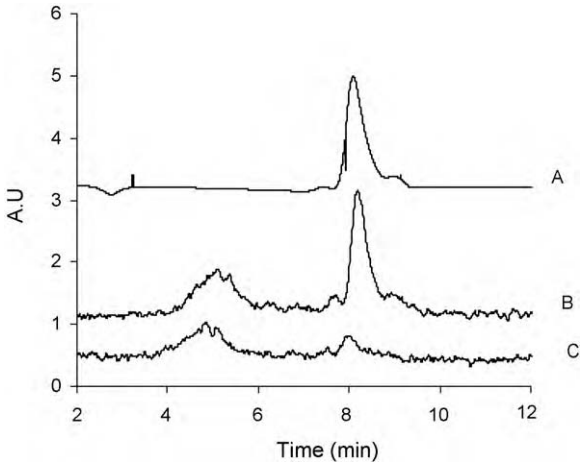

Fig. 3. Chromatograms obtained for (A) Chl *a* standard solution (1 $\mu g\,L^{-1}$), (B) spiked seawater sample (1 $\mu g\,L^{-1}$) and (C) seawater sample with the proposed procedure.

Table 2
Optimum conditions for selective determination of chlorophyll a with the proposed procedure.

	Optimum experimental conditions
Taken standard or samples concentration	0.1–1 $\mu g\,L^{-1}$ (injected concentration 1–10 $\mu g\,L^{-1}$)
Processed volume of standard or samples	10 mL
Extraction procedure	Nylon membrane filter. Extraction solvent 1 mL ethanol
IT-SPME	Capillary column: TRB-5: PDMS 70 cm (50 μL). 0.32 internal diameter, 3 μm PDMS coating
Chromatographic conditions	Analytical column: Zorbax SB C_{18} (15 cm × 0.5 mm (5 μm particle size) Isocratic elution mode: (95:5)% ethanol:water 20 $\mu L\,min^{-1}$

3.2. Study of the preconcentration device

TRB-5, a non-polar coating, was selected for the capillary column taking into account the low polarity of Chl *a* and the nature of the extraction solvent (ethanol). The retention of Chl *a* in the capillary column was helped by a dilution of the analytes with 10% water previous to the injection. This water content introduced an increase on the polarity of the medium in which chlorophylls [20] were solved and so a higher retention in the PDMS coating was achieved.

We studied the volume of the loop (capillary length) and the processing volumes which were varied between 18–50 μL (25–70 cm) and 25–100 μL, respectively. Fig. 2 shows the normalized area as function of the loop size, as can be seen the injection of 50 μL in a 50 μL (70 cm in length) loop provided the highest analytical signal (peak area) for the estimation of Chl *a* at low ppb levels. Higher processing volumes of sample resulted in the autoelution of Chl *a* from the capillary. Fig. 3 shows the chromatogram obtained for Chl *a* with a retention time of 8.6 min. Under these conditions, Chl *b* did not interfere in the determination of Chl *a* as it is eluted at 6.9 min.

Table 3
Precision obtained with the IT-SPME Capillary LC.

Concentration ($\mu g\,L^{-1}$)	RSD(%)	
	Intraday ($n=3$)	Interday ($n=6$)
0.09	2	11
0.30	2	10
0.40	7	9

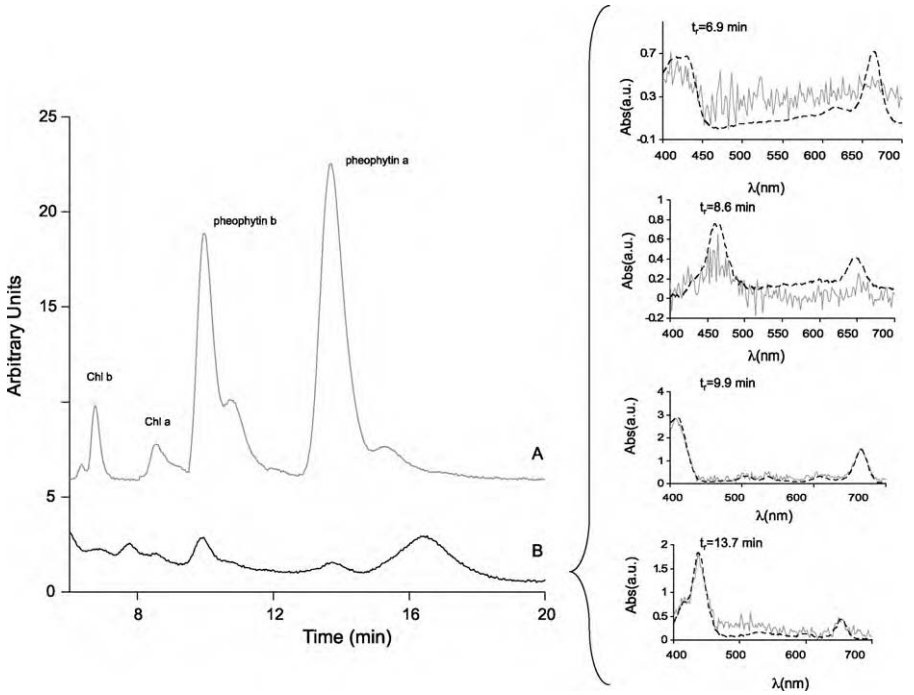

Fig. 4. Left: chromatograms obtained for the (B) wastewater sample and (A) mixture of pheophityn *a* and pheophityn *b* (1 mg L^{-1}) generated from a mixture of Chl *a* and Chl *b* (1 mg L^{-1}) (for more details see Section 2). Right: spectra of the sample obtained at retention times (t_r) 6.9, 8.6, 9.9 and 13.7 min. Doted line spectra at t_r 6.9, 8.6, 9.9 and 13.7 min corresponds to Chl *b*, Chl *a*, pheophityn *b* and pheophityn *a* spectra in standard solutions.

3.3. Analytical parameters

The calibration equation obtained at the optimum conditions shown in Table 2 was $y = b_0 + b_1x$, where b_0 (s_{b0}) = 7(1) and b_1 (s_{b1}) = 37(1); x was expressed in μg L^{-1} of Chl *a* in the sample; R^2 = 0.9990 and n = 6.

Detection limit (LoD) was experimentally determined as the analyte concentration that produced a signal-to-noise of 3, being 0.05 μg L^{-1}. The LoD achieved with IT-SPME Capillary LC procedure was clearly an advantage of this procedure as the concentration levels were satisfactory for the analysis of Chl *a* in water samples without off-line preconcentration. Besides, this LoD value was also better than those previously reported in the literature [12].

Precision was also evaluated by processing standard solution with the overall procedure: extraction of the pigment from the nylon filter and IT-SPME coupled to Capillary LC. Intraday RSD values were calculated by injecting three consecutive replicates of a standard of Chl *a* (0.5 μg L^{-1}) and the interday RSD were calculated by injecting the same standard solution during six consecutive days. Intra and interday relative standard deviations (RSD) are shown in Table 3. As can be seen, satisfactory intra and interday RSD values were obtained with this procedure.

The selectivity of the proposed procedure was evaluated with other chlorophylls (chlorophyll *b*, Chl *b*) and chlorophyll degradation products, pheophityn *a* and pheophityn *b* for Chl *a* and Chl *b*, respectively. Chlorophylls degradation products can constitute an important fraction of the total content of pigments in some water samples Both, pheophytins *a* and *b*, were generated by acidification of solutions of Chl *a* and Chl *b* (see Section 2), respectively. Fig. 4A depicts the chromatogram obtained for pheophytin *a* and pheophytin *b* (1 mg L^{-1}) generated from a mixture of Chl *a* and Chl *b* (1 mg L^{-1}). High conversion factors of chlorophyls in pheophytins were achieved (see Fig. 4A). As can be seen, the proposed procedure was selective for Chl *a* determination as the other compounds eluted at different retention times.

3.4. Analysis of samples from different sources

The content of Chl *a* in water samples depends on the source. We analysed seawater and wastewater from a treatment plant. The content of Chl *a* in the seawater samples were near the LoD (0.05 μg L^{-1}). Fig. 3 shows the chromatograms obtained for the seawater sample and spiked seawater sample (1 μg L^{-1}). A recovery study at different concentration levels was carried out. The recoveries obtained for added concentrations of 0.09, 0.30 and 0.40 μg L^{-1} were, 77 ± 8, 90 ± 10 and 73 ± 7%. As it was expected, satisfactory results were obtained for all the analysed samples.

Finally, we analysed the wastewater, the found concentration of Chl *a* was 1.30 ± 0.02 μg L^{-1}. Fig. 4B shows the chromatogram obtained for this water sample. In this water sample, we observed the presence of Chl *b* (t_r 6.9 min). Fig. 4B (right) shows the spectra obtained at retention times 6.9 and 8.6 min, respectively. The comparison of the spectra at these retention times confirmed the presence of Chl *a* and Chl *b* in this samples as they completely match with the spectra of standards of Chl *a* and Chl *b* (dotted lines). In addition, comparison of Fig. 4A and B revealed the existence of pheophityns *a* and *b* in this water sample. The proposed procedure allowed the identification of these derivates as they eluted at 9.9 and 13.7 min. Spectra at these retention times are also shown in Fig. 4. The match between the spectra of the sample and the standards of pheophityns *a* and *b* (dotted line) was satisfactory.

Therefore, the extraction with nylon membrane filter combined with the IT-SPME Capillary LC system could be used for the analysis of water samples with good extraction efficiency and accurate results.

4. Conclusions

The present work establishes a direct extraction procedure for extracting chlorophyll *a* from water samples. Moreover, the extraction method based on the use of nylon membrane filters for Chl *a* uses a low volume (1 mL) of a non-hazardous solvent (ethanol),

minimizes the sample volume (10 mL) and analysis time in reference to published method [12]. Also, a novel approach combining IT-SPME and Capillary LC with diode array detection, has been proposed for the selective determination of Chl *a* at low concentration levels in water samples.

The global analytical method has been developed using ethanol as unique solvent for the extraction procedure and the chromatographic separation. The proposed methodology resulted in satisfactory linearity in the working range, good LoD and suitable precision and accuracy.

An environmental friendly procedure was developed that allows the automation of the overall procedure for the estimation of Chl *a* as a good indicator of phytoplankton biomass. Miniaturization is used as tendency of analytical chemistry.

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