



Pulsed amperometric detection: Extending the applicability of HPLC to substances with two-step redox processes

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ABSTRACT

Electrochemical detection coupled to liquid chromatography provides high selectivity compared to optical detection due to the lower number of electrochemically active compounds. Nevertheless, not all electrochemically active substances may be detected with the classical amperometric detection that involves the application of a single potential. For instance, in flow conditions, the main biomarkers of cinnamon (eugenol, coumarin, cinnamaldehyde and cinnamic acid) require an oxidation step prior to their amperometric detection. Pulsed amperometric detection (PAD) is proposed to address the electrochemical determination of substances with such complex redox behaviour in flow conditions because it allows the application of multiple potential steps that mimic the sequential stages that would be performed in static voltammetric measurements. PAD waveform has been optimized resulting in two scenarios ($t_{ox} \gg t_{red}$ and $t_{ox} \ll t_{red}$) with different detection mechanisms involved that lead to either upward or downward peaks. In both scenarios, a notable analytical performance was achieved for all considered compounds and the proposed method was successfully validated for the analysis of a cinnamon sample, obtaining statistically comparable results to those obtained by HPLC-UV. The HPLC-PAD approach proposed in this work will extend the applicability of HPLC-ED to a wider set of substances that include not only those compounds that can be determined by a direct oxidation or reduction process but also those that require a two-step redox process. This will have a positive impact on targeted food analysis, in which it will facilitate the determination of electrochemical biomarkers in the presence of complex matrix samples.

1. Introduction

The analysis of food products, together with the detection of food fraud and the assurance of food safety, has traditionally been carried out by means of separation techniques such as liquid chromatography (LC) or gas chromatography (GC). This is mainly due to the complex nature of food matrices, which present a wide range of volatile, semi-volatile or non-volatile compounds [1–4]. In the case of liquid chromatography, it can be coupled to different detectors. Among the more classical detection methods are ultraviolet detection (UV) or fluorimetric detection (FD), while more advanced and modern detectors include mass spectrometry (MS) [5–8]. In particular, LC-UV offers good stability and sensitivity for light-absorbing compounds; however, due to the complex matrices and composition of food products, the resulting chromatograms can be intricate and difficult to interpret [9]. In contrast, electrochemical detection (ED) provides higher selectivity, as it only detects electrochemically active compounds. This results in simpler

chromatograms, thereby facilitating the characterization and targeted analysis of complex food samples in which the main biomarkers are electroactive [6,10–12].

In recent decades, the availability of commercial screen-printed electrodes (SPEs) that can be mass-produced in a reproducible manner and at a relatively low cost has facilitated the coupling of HPLC with ED because, unlike traditional electrodes like glassy carbon, SPEs do not require surface polishing before each use. Furthermore, advancements in the ink industry have enabled the fabrication of SPEs from a variety of materials, including carbon, carbon nanoallotropes, gold and silver [13–15]. Additionally, the integration of a three-electrode configuration (working, counter and (pseudo)reference electrodes) into a single strip results in simpler electrochemical cells that require minimal sample volume and are easily coupled to an HPLC system [12,16,17].

ED in chromatographic methods includes several detection techniques, such as amperometry, coulometry, voltammetry, and potentiometry [18–20]. Among these, amperometric detection is one of the

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most widely used. This technique involves applying a constant oxidative or reductive potential and measuring the response current over time. However, it has certain limitations, since only the compounds that undergo redox reactions at the applied potential can be detected, and some analytes may adsorb on the electrode surface, hindering further detection [21–23]. To address these limitations, pulsed amperometric detection (PAD) can be employed. PAD allows the application of cleaning potentials to remove adsorbed species in the case of blocking of the working electrode [24] and enables the application of multiple potentials in a periodic sequence. This could be particularly advantageous for detecting analytes with a complex redox behaviour that implies several oxidation or reduction steps. For instance, the initial PAD measurements reported in the literature based on gold electrodes could be carried out under different modes: direct mode follows the faradaic response of the analyte, whereas indirect mode records the inhibition of the non-faradaic response, which was typically associated with the anodic formation of oxide on the surface of a gold electrode [25]. Thus, the resulting signal obtained by PAD is characteristic of the analyte and the detection mode employed [22,26–28].

Therefore, this work aims to explore the possibilities to extend the applicability of HPLC-ED to the determination of a wider set of electroactive compounds by targeting electroactive substances that cannot be directly detected by the application of a single potential. In particular, our study focuses on analytes that, when measured by voltammetry in static conditions, would require a previous oxidation or reduction step prior to the potential scan (i.e. as in stripping voltammetry). This strategy cannot be directly transferred to classical amperometric detection, which is crucial to provide the shorter response time required for an electrochemical detection in flow conditions such as the one employed in HPLC. For this purpose, the application of different pulses of PAD and the influence of each parameter were investigated as a novel strategy to address the determination of analytes with complex redox behaviour. As a case of study, the main biomarkers for the authentication of different species of cinnamon (coumarin, eugenol, cinnamaldehyde and cinnamic acid) [29] were selected since they present this type of redox behaviour. Furthermore, the determination of cinnamon biomarkers will also allow us to assess the performance of HPLC-PAD in complex matrices such as those usually found in food authentication.

2. Experimental

2.1. Chemicals and samples

For the preparation of the mobile phase, Milli-Q water (reference A+, Millipore, France), formic acid (98 % PanReac AppliChem, Barcelona, Spain), and methanol (HPLC Gradient rate, ≥ 99.9 %; Fisher Scientific, Geel, Belgium) were employed. The standards of cinnamic acid and cinnamaldehyde were provided by Thermo Fisher (Kandel, Germany), while eugenol and coumarin were supplied by Sigma Aldrich (Steinheim, Germany). A concentrated standard stock solution was prepared for each compound at a concentration of 1000 mg L^{-1} in methanol and stored at 4°C . Intermediate solutions were prepared weekly by appropriate dilution with methanol and stored at 4°C .

2.2. Differential pulse voltammetry

Differential pulse voltammetric (DPV) measurements were performed on an Autolab PGSTAT12 potentiostat (EcoChemie, The Netherlands) and coupled to a Metrohm 663 VA Stand (Metrohm, Switzerland). The instrument was controlled with a computer with GPES 4.9 software (version 4.9.007 available for Windows XP, Ecochemie, The Netherlands). Electrochemical measurements were carried out in a conventional cell with 3 electrodes (working, reference and auxiliary). The working electrode of the SPCE DRP-110 from Metrohm-Dropsens (Spain) was connected to the potentiostat by means of a flexible cable. External Ag/AgCl (3 M KCl) and platinum electrodes were provided by

Metrohm and used as reference and auxiliary electrodes, respectively. An oxidative pretreatment was carried out by applying $+1 \text{ V}$ for 30 s under stirring. Then, the potential was scanned from an initial potential of $+1 \text{ V}$ to a final potential of -1.9 V . This potential scan was performed with a step potential of 12.5 mV , pulse amplitude of 50 mV and pulse time of 50 ms .

2.3. HPLC-ED analysis

High performance liquid chromatography with electrochemical detection (HPLC-ED) analysis was achieved on a chromatographic Agilent 1260 Infinity instrument (Palo Alto CA, USA), which comprised a quaternary pump VL with a vacuum degasser integrated (G7111A) and a manual injector (G1328C) controlled by an instant pilot (G4208A). The reverse-phase separation was performed in a Kinetex® column ($5 \mu\text{m}$ C18 $100 \text{ \AA}$, $100 \times 4.6 \text{ mm}$) supplied by Phenomenex (Torrance, CA, USA). The separation was performed under an isocratic elution mode using 65:35 0.1 % aqueous formic acid: methanol. In-between measurements, the content of methanol was raised to 95 % to ensure that all other components of cinnamon samples were eluted, and then the column was re-equilibrated at 35 % methanol. The flow rate was 1 mL min^{-1} , and the injection volume was $10 \mu\text{L}$.

Electrochemical detection was performed with an HPLC electrochemical cell for SPEs supplied by Metrohm-Dropsens (ref. HPLCELL). The employed SPEs were screen-printed carbon electrodes (SPCE) provided by Metrohm-Dropsens (ref. DRP-110), which comprise a three-electrode configuration with a carbon disk of 4 mm as a working electrode, a carbon-ink auxiliary electrode, and a silver pseudo-reference electrode. SPCEs were coupled through a flexible cable (ref. CAC, Metrohm-Dropsens) to an Autolab PGSTAT 12 potentiostat by EcoChemie. The integration of chromatographic peaks was also performed using GPES software.

Unless otherwise stated, two different pulsed amperometric detection (PAD) waves were employed for HPLC-PAD. The first wave ($t_{\text{ox}} \gg t_{\text{red}}$) consisted in pulses of 0.1 s at -1.5 V and 1.25 s at $+1 \text{ V}$; measurements were taken at -1.5 V . The second wave ($t_{\text{ox}} \ll t_{\text{red}}$) consisted in pulses of 1.25 s at -1.5 V and 0.1 s at $+1 \text{ V}$; measurements were taken at the last 0.1 s of each reduction step (-1.5 V). Given their disposable character and in order to ensure reliable measurements, each SPCE unit was stabilized for 10 min before being used to record a blank signal and a sample injection.

2.4. Sample and sample treatment

A sample of Ceylon cinnamon, purchased in a local store, was prepared by dissolving 0.5 g of the powdered sample in 2 mL of methanol and vortexing for 1 min. Then the extract was sonicated for 15 min and centrifuged for 45 min at 4500 rpm . The supernatant was collected, filtered through $0.22 \mu\text{m}$ nylon filters, and methanol was added to a final weight of 2 g . The sample was prepared in triplicate and stored at -18°C until analyzed.

For comparison purposes, the sample was also analyzed using an HPLC-UV method previously optimized by Pages-Rebull et al. [30]. The method employed a gradient elution program combining 0.1 % formic acid in Milli-Q water (solvent A) and methanol (solvent B) as follows: 0–25 min, linear gradient from 5 to 95 % solvent B; 25–30 min, isocratic elution at 95 % solvent B; 30–33 min, from 95 to 5 % solvent B; and 33–35 min, re-equilibration at 5 % solvent B. Chromatographic detection was carried out at 280 nm , with an injection volume of $10 \mu\text{L}$ and a mobile phase flow rate of 1.0 mL min^{-1} . HPLC-UV analysis was performed using an Agilent 1200 chromatographic system (Palo Alto, CA, USA), comprising a vacuum degasser (G1322A), a quaternary pump (G1311A), an autosampler (G1329A) and an ultraviolet-visible detector (G1314). Separation was achieved using the same Phenomenex Kinetex® reversed-phase C18 column as employed in the HPLC-ED.

3. Results and discussion

3.1. Electrochemical detection

The HPLC-ED determination of cinnamaldehyde, cinnamic acid, eugenol and coumarin as important bioactive compounds in cinnamon characterization and authentication was first attempted through the application of a single and constant positive (oxidation) or negative (reduction) potential, considering that their chemical structure suggests that these compounds should be susceptible to electrochemical processes. Nevertheless, as can be seen in Fig. S1, initial attempts to detect them through HPLC-ED yielded no results as no compounds were detected by the application of a reductive potential (i.e. -1 V) and only eugenol provided a peak in oxidative potentials (i.e. $+1$ V). It must be noticed that in both cases, there is a baseline quite different from zero. This suggests a constant background process of the mobile phase, which is reduced at -1 V (negative current around -50 μ A in Fig S1a) and oxidized at $+1$ V (positive current around 0.5 μ A in Fig S1b). The comparison of such currents shows that the reduction of the mobile phase is far more important than its oxidation at the potentials considered. Taking into account the potentials considered, we hypothesize that the oxidation of the mobile phase should consist in water yielding oxygen, and its reduction should be due to water generating hydrogen.

In this context, where no peaks are obtained in HPLC-ED, HPLC-PAD appears as an alternative approach to carry out the electrochemical detection. Nevertheless, the direct application of PAD is particularly difficult when one lacks information regarding the electrochemical process of the involved compounds. Thus, although the electrochemical processes in static conditions are not directly translated to flow conditions in thin layer cells as the one used in HPLC-ED (transport regime and other experimental conditions are completely different), the electrochemical behaviour of the four considered compounds in aqueous buffer solution was assessed by means of DPV in static conditions in order to obtain preliminary parameters to start optimizing PAD conditions. In this case, in terms of peak definition, the best results were obtained with the introduction of an oxidative pretreatment ($+1$ V during 30 s) prior to a reduction scan from $+1$ V to -1.9 V, where a clear reduction voltammetric peak was observed for all analytes, as it is shown in Fig. S2. The obtained voltammetric peak didn't increase with more positive oxidative pretreatments and nor with longer oxidation times, which suggests that the compounds are not being adsorbed on the electrode surface. In contrast, the application of a reductive pretreatment before an oxidation scan didn't produce any voltammetric signal.

The translation of this DPV method in static to flow conditions cannot be implemented through HPLC-ED (with classical amperometric detection) using a single SPCE because classical HPLC-ED is based on the application of a single and constant potential, either positive or negative. In contrast, PAD is an alternative electrochemical approach that, while maintaining the required fast response in flow conditions, allows the introduction of multiple potential steps. Such steps, however, have to be short enough to allow several repetitions of the step program during the elution of the analyte along the electrode surface. In this work, analytes are passing by the electrode during ca. 30–180 s, and every cycle of oxidation pulse + reduction pulse should last no longer than 1–2 s to allow at least 15 cycles per analyte/chromatographic peak. Therefore, HPLC-PAD is a promising strategy to allow the electrochemical determination in flow conditions of organic compounds that require an oxidation or reduction step prior to their detection.

Thus, the detection of cinnamaldehyde, cinnamic acid, eugenol and coumarin was studied by HPLC-PAD in isocratic elution mode using a mobile phase of 0.1 % aqueous formic acid: methanol (65:35). For this purpose, the shape of the PAD wave was first optimized considering two potential values: (i) $+1$ V to replicate the preoxidation step, and (ii) -1.5 V, negative enough to reduce all the previously oxidized species, according to Fig. S2. In this sense, oxidation and reduction times (t_{ox} , t_{red}) were evaluated considering several values and ratios, which leads to

distinct shapes of the PAD signal. Measurements were always acquired during 0.1 s at -1.5 V, and the time at which the measurement took place (t_{mes}) was also optimized along the duration of the reductive step. A schematic representation of the PAD wave and the optimized parameters is shown in Fig. 1a. During the optimization process, it was observed that the chromatographic profile displayed great differences depending on the applied t_{ox} , t_{red} and t_{mes} , to the point that peaks shifted from upward to downward directions and even disappeared in some combinations. Fig. 1b shows an example of three different PAD waves that lead to different peak profiles.

A plausible explanation of this phenomena is that, in the absence of analyte (only mobile phase arrives to the SPCE), a negative baseline is detected and its intensity value depends on the shape of the PAD wave: (i) when $t_{ox} \gg t_{red}$, the mobile phase is oxidized most of the time and, when measurements are taken at -1.5 V, a large reduction current is generated by the products of the previous oxidation, leading to very negative baselines; (ii) when $t_{ox} \ll t_{red}$, the mobile phase is oxidized just for a short time and, when measurements are taken at -1.5 V, a low reduction current is obtained from the scarce products of the previous oxidation, which leads to less negative baselines. This can be observed in Fig. 1b, in which the blue signal recorded at $t_{ox} \gg t_{red}$ appears at much lower intensities than the orange signal recorded at $t_{ox} \ll t_{red}$. The yellow curve reflects an intermediate situation.

In the presence of analyte, when $t_{ox} \gg t_{red}$, both the analyte and the mobile phase are easily oxidized at $+1$ V. Therefore, when the signal is measured at -1.5 V, a reduction signal associated to both baseline and analyte is obtained and, consequently, a downward peak is observed when the analyte arrives to the SPCE (blue signal in Fig. 1b). As it can be seen in Fig. 2a for cinnamaldehyde, as the difference between t_{ox} and t_{red} increases, the intensity of the obtained downward peaks also increases. Similar behaviour was observed for all the considered analytes. On the other hand, as the analyte concentration increases, the contribution of the analyte to the measured signal is higher, and the peak increases proportionally. Thus, measurements taken in these conditions are equivalent to PAD measurements taken in direct mode, where the faradaic response of the analyte is the predominant factor.

In contrast, when $t_{ox} \ll t_{red}$, the time spent at $+1$ V is not long enough to oxidize both the analyte and the mobile phase. In baseline conditions, only the mobile phase reaches the SPCE and is oxidized, but when the analyte arrives to the SPCE, it is oxidized at the expense of the mobile phase. If the signal associated to the reduction of the analyte does not compensate the loss of reduction signal associated to the mobile phase, a less negative current is observed while the analyte is passing by the electrode, leading to an upward peak (orange signal in Fig. 1b). As it was observed in the previous scenario, the intensity of all peaks also increases as the difference between t_{ox} and t_{red} increases (Fig. 2b for cinnamaldehyde). As the analyte concentration increases, the loss of signal associated to the mobile phase is larger and the peak increases proportionally. Therefore, measurements taken at $t_{ox} \ll t_{red}$ behave as indirect PAD mode measurements that are based on both the faradaic response of the analyte and the decrease of the response of the mobile phase. In other words: long oxidation periods allow the oxidation/reduction of both mobile phase and analyte at maximum rate and their negative signals add up, whereas short oxidation pulses create a competition between analytes and mobile phase in the oxidation/reduction process taking place as they pass by the electrode, so that every analyte generates a decrease of the negative background in the form of an upward peak.

In an intermediate situation where $t_{ox} \approx t_{red}$, both processes take place at similar extent and their effects are counteracted. Thus, the loss of signal associated to the mobile phase is similar to the gain of signal associated to the analyte reduction, and no peak is observed (yellow signal in Fig. 1b). In this context, the moment in which the measurement is taken (t_{mes}) is crucial in order to define which of the two processes prevails. High t_{mes} favour the electrochemical processes of the analyte as compared to those of the mobile phase. Fig. 3a shows, for

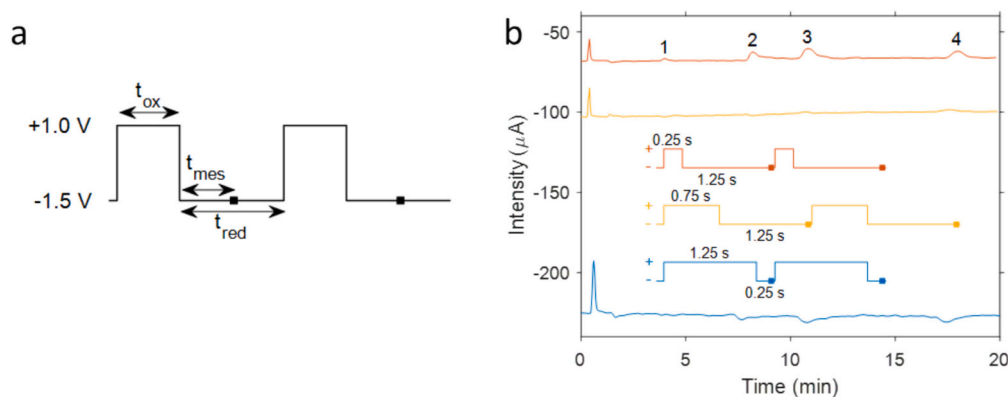


Fig. 1. (a) Representative waveform for pulsed amperometric detection. (b) HPLC-PAD signals of coumarin (1), cinnamaldehyde (2), cinnamic acid (3), and eugenol (4) obtained using the different PAD waves shown in the figure.

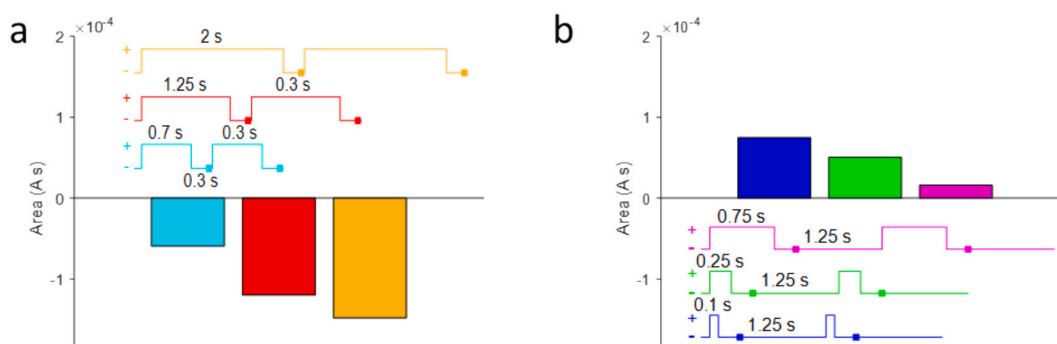


Fig. 2. (a) Area of the peak of cinnamaldehyde for different PAD waves with a fixed $t_{\text{red}} = 0.3$ s, and $t_{\text{ox}} = 0.7$ s (cyan), 1.25 s (red), 2.0 s (orange) and measuring at the end of t_{red} (0.2–0.3 s). (b) Area of the peak of cinnamaldehyde for different waveforms with a fixed $t_{\text{red}} = 1.25$ s, and $t_{\text{ox}} = 0.1$ s (blue), 0.25 s (green), 0.75 s (magenta) and measuring at $t_{\text{mes}} = 0.2$ –0.3 s. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cinnamaldehyde as an example, the effect of t_{mes} for two PAD waves with intermediate t_{ox} and t_{red} values. For instance, at small t_{mes} the suppression of the baseline (upward peaks) predominates, while as t_{mes} increases, the contribution of the analyte gains importance. This translates into upward peaks at small t_{mes} that gradually decrease as t_{mes} increases and may even switch to downward peaks for large values of t_{mes} . It should be pointed out that in the other two scenarios ($t_{\text{ox}} \gg t_{\text{red}}$ and $t_{\text{ox}} \ll t_{\text{red}}$), peak intensity always benefited from large t_{mes} . This can be observed for cinnamaldehyde as an example in Fig. 3b for $t_{\text{ox}} \gg t_{\text{red}}$ and Fig. 3c for $t_{\text{ox}} \ll t_{\text{red}}$.

3.2. Analytical performance

In view of the above-mentioned results, large peak intensities can be obtained at large t_{mes} in the two extreme situations ($t_{\text{ox}} \gg t_{\text{red}}$ and $t_{\text{ox}} \ll t_{\text{red}}$), which are based on different processes. Thus, both processes were further studied as suitable analytical signals for the determination of cinnamaldehyde, cinnamic acid, eugenol and coumarin. The particular parameters selected for each case (insets in Fig. 4), considering an oxidation potential of +1 V and a reduction potential of −1.5 V, were: (i) $t_{\text{ox}} = 0.1$ s, $t_{\text{red}} = 1.25$ s, $t_{\text{mes}} = 1.15$ –1.25 s for $t_{\text{ox}} \ll t_{\text{red}}$; and (ii) $t_{\text{ox}} = 1.25$ s, $t_{\text{red}} = 0.1$ s, $t_{\text{mes}} = 0$ –0.1 s for $t_{\text{ox}} \gg t_{\text{red}}$. As an example, the chromatograms obtained for a solution containing 20 mg L^{−1} and 150 mg L^{−1} of cinnamaldehyde, cinnamic acid, eugenol and coumarin for $t_{\text{ox}} \ll t_{\text{red}}$ and $t_{\text{ox}} \gg t_{\text{red}}$ conditions, respectively, are displayed in Fig. 4. In both cases, four well-defined upward (Fig. 4a) or downward (Fig. 4b) peaks are observed.

The analytical performance was evaluated in terms of sensitivity, linear range, linearity, limits of detection (LOD) and quantification

(LOQ), and reproducibility under the conditions specified in Section 2.3. The obtained performance is displayed in Table 1. In general, for all studied analytes, two linear ranges with good linearity were found using both PAD waves. The only exception was coumarin, which only showed one linear range at $t_{\text{ox}} \gg t_{\text{red}}$. The first linear range was relatively narrow (up to 24 mg L^{−1} for coumarin) for all analytes and, comparing both PAD waves, eugenol and cinnamic acid presented similar intervals, while for coumarin and cinnamaldehyde the intervals obtained at $t_{\text{ox}} \gg t_{\text{red}}$ were wider. The second linear range was significantly wider (about two times) for $t_{\text{ox}} \gg t_{\text{red}}$ for all analytes. Regarding sensitivities of the first linear range, which correspond to the slope of the calibration curve, cinnamic acid and coumarin showed, respectively, the highest and lowest sensitivities in both PAD waves. The effect of PAD wave on sensitivity was not homogeneous: (i) cinnamic acid and eugenol showed better sensitivity at $t_{\text{ox}} \gg t_{\text{red}}$; (ii) cinnamaldehyde sensitivity was similar in both cases; and (iii) coumarin showed a higher sensitivity at $t_{\text{ox}} \ll t_{\text{red}}$. Finally, LODs between 0.1 and 2.1 mg L^{−1} were achieved depending on the considered analyte and PAD wave. In addition, reproducibility in-between SPCEs was evaluated at two different concentration levels (10 and 75 mg L^{−1}), achieving %RSD ($n = 3$) lower than 7 % in all cases (Table 2). The good reproducibility values obtained allow the comparison of results obtained using different SPCEs units, which takes advantage of the benefits associated to the use of low-cost disposable SPCE (e.g., lack of electrode fouling, lack of cross contamination, no need of tedious polishing procedures...).

At the view of the obtained results, it seems that the PAD wave with $t_{\text{ox}} \gg t_{\text{red}}$ provides a better analytical performance for most of the analytes except for coumarin. Considering that $t_{\text{ox}} \gg t_{\text{red}}$ is associated to a direct PAD mode where the faradaic response of the analyte is the

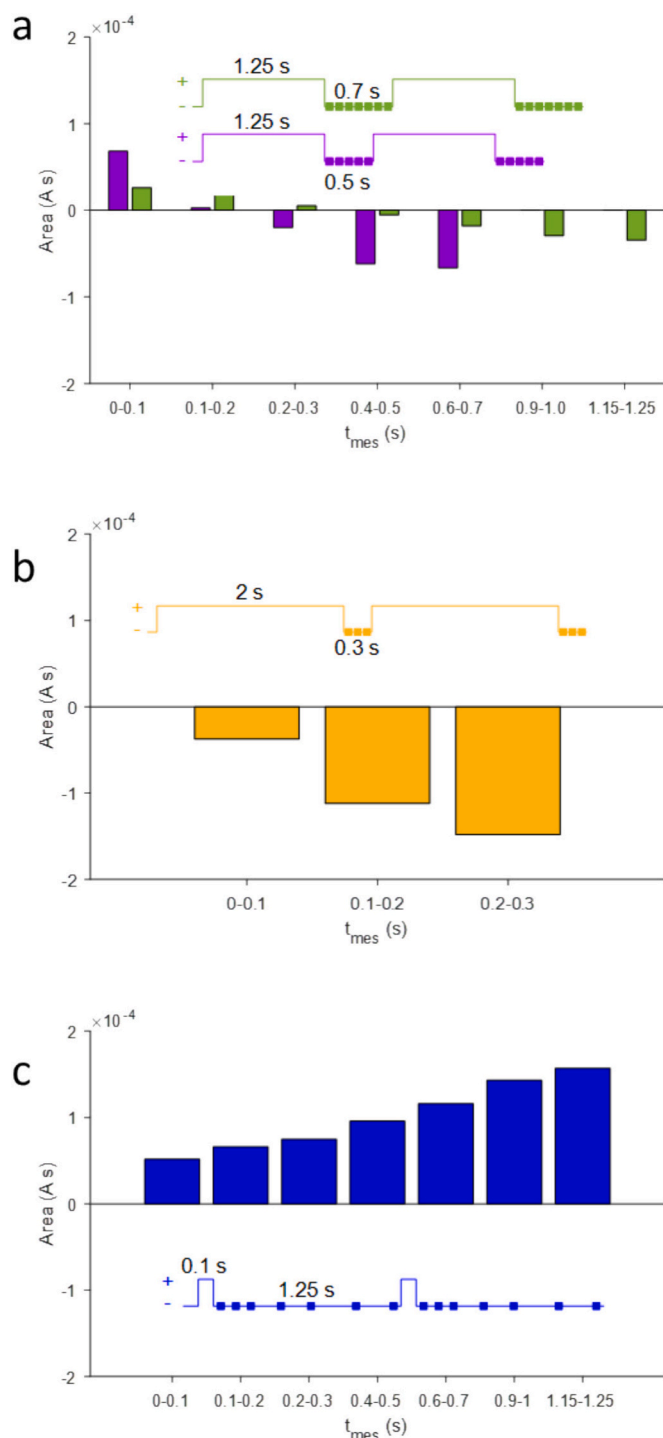


Fig. 3. Effect of measuring time (t_{mes}) in the signal obtained for cinnamaldehyde (peak area) using three types of PAD waves: a) $t_{ox} \approx t_{red}$, b) $t_{ox} \gg t_{red}$ and c) $t_{ox} \ll t_{red}$.

predominant factor, it makes sense that a better performance will be obtained for analytes showing a stronger electrochemical response. In contrast, coumarin, which is the less electroactive compound, shows a better analytical performance at the PAD wave with $t_{ox} \ll t_{red}$, which behaves as indirect PAD mode measurements that are based on both the response of the analyte and that of the mobile phase.

Comparing the results obtained in the evaluation of the analytical performance with other methods reported in the literature based on HPLC coupled to different detectors (mainly UV and MS, see Table S1) it

can be observed that the proposed method constitutes a competitive alternative. In terms of reproducibility, the results obtained are in line with other methods reported and, although the LODs may be higher than those reported for HPLC-UV or HPLC-MS, they are still adequate for cinnamon analysis. Furthermore, the linear ranges obtained with this method are more homogeneous between analytes, which facilitates the simultaneous determination of all analytes in real samples.

3.3. Sample analysis

To assess the applicability of the optimized HPLC-PAD strategy and its use for the analysis of complex food matrices, the determination of cinnamaldehyde, cinnamic acid, eugenol and coumarin in a cinnamon sample was carried out. According to literature, these four compounds are the main biomarkers for cinnamon authentication, allowing the discrimination between the most prominent two species of cinnamon: Ceylon cinnamon (*Cinnamomum verum*), which is the one with the most health benefits and higher price; and cassia cinnamon (*Cinnamomum cassia*), a lower price variety that also contains higher content of the hepatotoxic coumarin [31–33]. The usual concentrations of cinnamaldehyde, cinnamic acid, eugenol and coumarin in cinnamon samples [29] can be easily determined with the analytical performance demonstrated by the proposed HPLC-PAD strategy.

For the determination of cinnamaldehyde, cinnamic acid, eugenol and coumarin by HPLC-PAD, a standard addition calibration method was carried out in triplicate for a Ceylon cinnamon sample, in which the amount of coumarin is expected to be lower. For this purpose, the extracted cinnamon sample and three extracted samples fortified at increasing concentration levels of all four analytes were injected, and the corresponding chromatograms were recorded. This procedure was carried out using the two PAD waves considered in the previous section ($t_{ox} \gg t_{red}$ and $t_{ox} \ll t_{red}$). As it can be observed in Fig. S3, the profiles obtained for the extracted sample are not significantly altered by the presence of other substances in cinnamon samples, with the four studied analytes and an unidentified peak at ca. 19 min being the most prominent signals observed. The results obtained in the quantitative analysis are shown in Table 3, together with the concentrations determined by HPLC-UV for comparison purposes.

As observed in Table 3, both PAD waves allow the determination of cinnamaldehyde, cinnamic acid and eugenol in the analyzed cinnamon sample, but only the wave with $t_{ox} \ll t_{red}$ allowed the determination of coumarin. This agrees with the results obtained for the analytical performance, which provided higher LOD and LOQ for coumarin using the PAD wave with $t_{ox} \gg t_{red}$. In order to validate the results obtained applying the newly proposed HPLC-PAD, a two-tailed t -test (equal variances) was performed to compare each PAD wave with HPLC-UV. In all cases, for a confidence level of 95 %, the t_{calc} (values between 0.09 and 2.63) was lower than $t_{stat} = 2.78$, and hence there were no statistically meaningful differences between both methods (accepting the null hypothesis).

At the view of the obtained results, it can be confirmed that the coupling of PAD with HPLC allowed the determination of cinnamaldehyde, cinnamic acid, eugenol and coumarin in samples with a complex matrix such as cinnamon. It should be noted that, even in this case in which a Ceylon cinnamon sample was analyzed, one of the PAD waves ($t_{ox} \ll t_{red}$) was sensitive enough to determine the really low contents of coumarin expected in Ceylon cinnamon. For cassia cinnamon, which has higher amounts of coumarin, it is expected that both PAD waves would be successful in its determination.

4. Conclusions

In this work, the coupling of PAD to HPLC is proposed to extend the applicability of HPLC-ED to chemical substances that cannot be determined with the application of a single positive or negative potential. This was evaluated for the determination of coumarin, cinnamaldehyde,

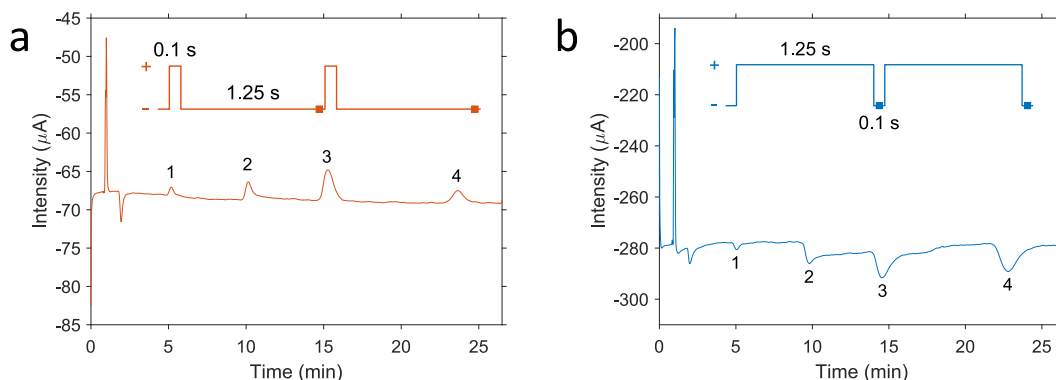


Fig. 4. HPLC-PAD signals obtained for a solution containing 20 mg L⁻¹ of coumarin (1), cinnamaldehyde (2), cinnamic acid (3) and eugenol (4) using a waveform with 0.1 s as t_{ox} and 1.25 s as t_{red} (a) and for a solution containing 150 mg L⁻¹ of each considered compound with 1.25 s as t_{ox} and 0.1 s as t_{red} (b).

Table 1

Analytical performance obtained by HPLC-PAD using two different PAD waves. For $t_{ox} < t_{red}$: $t_{ox} = 0.1$ s, $t_{red} = 1.25$ s and $t_{mes} = 1.15$ – 1.25 s; and for $t_{ox} > t_{red}$: $t_{ox} = 1.25$ s, $t_{red} = 0.1$ s and $t_{mes} = 0$ – 0.1 s. The standard deviations are denoted by parenthesis.

	$t_{ox} < t_{red}$				$t_{ox} > t_{red}$			
	Coumarin	Cinnamaldehyde	Cinnamic acid	Eugenol	Coumarin	Cinnamaldehyde	Cinnamic acid	Eugenol
1st interval								
Sensitivity ($\mu A s mg^{-1} L^{-1}$)	1.91 (0.04)	4.7 (0.1)	9.3 (0.4)	3.42 (0.09)	0.70 (0.03)	5.4 (0.2)	25.3 (0.9)	17.3 (0.6)
R ²	0.990	0.994	0.970	0.990	0.983	0.989	0.983	0.984
Linear range (mg L ⁻¹) ^a	1.4–12.0	0.4–2.6	2.2–10.5	1.8–12.5	7.1–24.0	1.9–10.4	2.4–10.5	2.7–12.5
LOD (mg L ⁻¹)	0.4	0.1	0.7	0.5	2.1	0.6	0.7	0.8
2nd interval								
Sensitivity ($\mu A s mg^{-1} L^{-1}$)	0.220 (0.009)	1.75 (0.03)	3.6 (0.1)	2.51 (0.08)	–	0.387 (0.006)	1.68 (0.04)	1.98 (0.04)
R ²	0.984	0.994	0.989	0.990	–	0.995	0.992	0.991
Linear range (mg L ⁻¹) ^a	12.0–90.0	2.6–78	10.5–78.8	12.5–93.8	–	10.4–156.0	10.5–157.5	12.5–187.5

^a Linear range with the LOQ as the lower value.

Table 2

Reproducibility values obtained by HPLC-PAD with three different SPCE units and using two different PAD waves. For $t_{ox} < t_{red}$: $t_{ox} = 0.1$ s, $t_{red} = 1.25$ s and $t_{mes} = 1.15$ – 1.25 s; and for $t_{ox} > t_{red}$: $t_{ox} = 1.25$ s, $t_{red} = 0.1$ s and $t_{mes} = 0$ – 0.1 s.

	$t_{ox} < t_{red}$				$t_{ox} > t_{red}$			
	Coumarin	Cinnamaldehyde	Cinnamic acid	Eugenol	Coumarin	Cinnamaldehyde	Cinnamic acid	Eugenol
Reproducibility (RSD,%)								
Level 1 (10 mg L ⁻¹)	3.7	5.4	7.0	6.2	6.8	2.1	4.4	5.7
Level 2 (75 mg L ⁻¹)	6.1	6.2	3.3	3.7	–	1.4	2.0	2.5

Table 3

Concentration of cinnamaldehyde, cinnamic acid, eugenol and coumarin (in mg kg⁻¹) obtained in the analysis of a Ceylon cinnamon sample using the two selected PAD waves as well as HPLC coupled to UV detection at 280 nm. The standard deviations are denoted by parenthesis.

	Coumarin	Cinnamaldehyde	Cinnamic acid	Eugenol
HPLC-PAD				
$t_{ox} < t_{red}$	8.0 (0.2)	3200 (100)	128 (2)	135 (3)
$t_{ox} > t_{red}$	< LOQ	3210 (60)	131.8 (0.9)	133 (1)
HPLC-UV	8.04 (0.05)	3290 (30)	127 (6)	135.1 (0.9)

cinnamic acid and eugenol, the main biomarkers of cinnamon. An initial assessment of the parameters that define the waveform of PAD showed the importance to properly define t_{ox} , t_{red} and t_{mes} . These parameters not only determine the intensity of the obtained signals but also the detection mechanism involved (faradaic response of the analyte or loss of signal associated to the mobile phase), which will define whether upward, downward or no peaks are obtained. The highest intensity was obtained for those PAD waves in which $t_{ox} > t_{red}$ (downward peaks) or $t_{ox} < t_{red}$ (upward peaks) and using large t_{mes} . For all the studied

compounds, both PAD waves provided an appropriate analytical performance, proving the suitability of the newly developed HPLC-PAD strategy for the determination of compounds with complex redox behaviour. The applicability of this was further validated for the analysis of cinnamon samples, allowing the determination of coumarin, cinnamaldehyde, cinnamic acid and eugenol with statistically comparable results to HPLC-UV with a 95 % confidence level. It should be highlighted that HPLC-PAD was able to determine the low levels of coumarin content in Ceylon cinnamon samples while providing a cleaner chromatogram, since only electroactive compounds of cinnamon will provide a signal in HPLC-PAD. Thus, HPLC-PAD would be a promising strategy not only for the determination of cinnamon biomarkers in particular, but also for the determination of other electroactive biomarkers commonly found in food. For this purpose, the versatility of PAD (where different parameters can be optimized) appears to be especially convenient to enhance the peaks of target analytes and minimize the effects of interfering signals and compounds.

CRediT authorship contribution statement

Josep Pages-Rebull: Writing – review & editing, Visualization,

Methodology, Investigation, Formal analysis. **Clara Pérez-Ràfols:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Conceptualization. **Núria Serrano:** Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Conceptualization. **José Manuel Díaz-Cruz:** Writing – review & editing, Visualization, Validation, Resources, Methodology.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2025.116086>.

Data availability

Data will be made available on request.

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