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A voltammetric approach for the quantification of thymoquinone in *Nigella Sativa* products

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Thymoquinone (TQ), a bioactive compound found in *Nigella sativa* seed oil, has been widely studied for its therapeutic potential. In this study, a new, sensitive, and cost-effective method was developed for TQ determination using square-wave voltammetry (SWV) with an environmentally friendly carbon paste electrode. Due to the morphology of the TQ oxidation signals, three calibration curve construction methods were tested, namely, based on: current height, peak area and cumulative voltammetry concept. The broadest linear range was established when calibration curve was constructed on the basis of TQ peak current height (LOD 8.9 nmol·L^{-1}, LOQ 29.8 nmol·L^{-1}). This method was validated through the analysis of real samples, including *Nigella sativa* seed oil and dietary supplement, with the results showing a strong correlation to those obtained by the HPLC reference method. The developed method represents a significant advancement in the electrochemical detection of TQ, offering practical benefits in terms of simplicity, precision, and cost-effectiveness.

Keywords Thymoquinone, Voltammetry, Carbon paste electrode, *Nigella sativa*, *Nigella sativa* seed oil, Black cumin

In today's rapidly advancing scientific landscape, the analysis of chemical compounds has become a cornerstone of research, with a significant number of analytical studies being published each year[1–3]. This trend underscores the growing interest in understanding the chemical constituents that impact human health and the environment. While many compounds exhibit beneficial effects, their concentrations must be carefully monitored to prevent adverse outcomes[4]. Accurate quantification is therefore crucial, particularly for compounds like thymoquinone (TQ), which has garnered considerable attention for its therapeutic potential[5].

Thymoquinone is a bioactive compound primarily found in the seeds of *Nigella sativa*, commonly known as black cumin. This compound is also present in several other medicinal plants. It has been identified in the essential oils of *Thymus vulgaris* (thyme) and *Origanum vulgare* (oregano), contributing to their well-documented antioxidant and antimicrobial properties[6,7]. Additionally, *Monarda fistulosa* (wild bergamot) and *Trachyspermum ammi* (ajwain) also contain high amounts of thymoquinone, further extending its relevance in traditional and modern medicinal applications[8–10]. Black cumin has been used for centuries in traditional medicine, particularly in Middle Eastern and South Asian cultures, due to its wide–ranging health benefits. Recent scientific research has validated many of these traditional claims, highlighting TQ's potent antioxidative, anti–inflammatory, and anticancer properties[11,12]. Thymoquinone's ability to scavenge free radicals and modulate oxidative stress pathways makes it a promising candidate for the treatment of various chronic diseases, including cardiovascular diseases, diabetes, and neurodegenerative disorders[13,14]. Additionally, TQ has demonstrated protective effects against liver and kidney toxicity, and it has been shown to possess antimicrobial activity against a broad spectrum of pathogens, including bacteria, fungi, and viruses[15,16].

Given its significant therapeutic potential, there is a strong need for accurate and reliable methods to determine thymoquinone concentrations in various matrices, such as food products, dietary supplements, and biological samples. High-performance liquid chromatography (HPLC) has long been the gold standard for TQ quantification due to its precision, sensitivity, and ability to separate TQ from other components in complex mixtures[16,17]. HPLC methods typically involve the use of ultraviolet (UV) detection, which allows for the direct

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monitoring of TQ at its characteristic absorption wavelength. However, despite its widespread use, HPLC can be time-consuming and requires sophisticated equipment, making it less accessible in resource-limited settings[18,19]. In addition to HPLC, other chromatographic techniques[5], such as high-performance thin-layer chromatography (HPTLC)[20] and gas chromatography-mass spectrometry (GC-MS)[21], have also been employed for TQ determination[22]. These methods offer high sensitivity and specificity[23] but are often limited by their complexity[24] and the need for extensive sample preparation[25–27]. Consequently, there is a growing interest in developing alternative analytical methods that are both simpler and faster, without compromising accuracy.

Voltammetric methods, particularly those based on the electrochemical reduction of thymoquinone, represent a promising alternative for its determination[28–30]. Electrochemical techniques are known for their simplicity, low cost, and ability to provide rapid results, making them ideal for routine analysis in both research and industrial settings[31,32]. Voltammetry, which involves measuring the current that flows in an electrochemical cell as a function of an applied voltage, can be used to study the redox behaviour of TQ and quantify its concentration in various samples[33,34]. This technique is particularly advantageous because it requires minimal sample preparation and can be applied directly to complex matrices, such as oils and biological fluids. Despite the well-established electrochemical behaviour of thymoquinone, all reported voltammetric methods rely exclusively on its reduction peak, with variations primarily based on supporting electrolyte conditions[30,35] Oxidation-based determination of thymoquinone has remained entirely unexplored in electroanalytical research, leaving a significant gap in available methodologies. This study introduces, for the first time, an oxidation-based voltammetric strategy, broadening the analytical framework for TQ detection and enhancing its practical applicability across diverse sample types[29,33,34,36].

In this study, we focus on developing a voltammetric method for the determination of thymoquinone using counterpart protonated hydroquinone oxidation processes, unreported in the literature before. To overcome the limitations of existing electrochemical methods, we developed a novel voltammetric strategy based on the oxidation of the protonated hydroquinone counterpart of thymoquinone. This previously unreported approach expands the range of electroanalytical tools available for TQ quantification. The method was optimized by systematically exploring different measurement parameters, including electrode material, electrolyte composition, and scan settings. A particular challenge was the interpretation of the complex voltammetric response, necessitating an in-depth evaluation of both classical and modern analytical techniques, including cumulative voltammetry. This refined approach enhances detection accuracy and provides an alternative means of quantification, particularly in cases where reduction-based methods face limitations. We have proposed and evaluated classic and novel approaches in delivering reliable and useful analytical data. Recently refreshed idea of cumulative voltammetry is here taken into cautious analysis and applied[37]. The optimized voltammetric procedure was then validated against HPLC measurements, ensuring the accuracy and reliability of the results. To demonstrate the practical applicability of the developed method, analysis of thymoquinone in real samples, including oil extracts from *Nigella sativa* and commercially available dietary supplements was performed. The comparative analysis with HPLC not only underscores the effectiveness of the proposed voltammetric approach but also highlights its potential as a routine analytical tool in quality control and research settings.

Through this work, we aim to provide a validated, accessible method for voltammetric thymoquinone determination that can be utilized in various fields, from food quality assessment to pharmacological research. By advancing the analytical techniques available for TQ quantification, we contribute to the broader effort of ensuring the safety and efficacy of products containing this valuable compound.

Experimental

Apparatus and instrumentation

Voltammetric measurements were performed using a μAutolab Type III potentiostat/galvanostat controlled by GPES software (version 4.9, Metrohm-Autolab, Netherlands). The investigations were carried out in a three-electrode system, with a silver/silver chloride electrode (3 mol·L^{-1} KCl, Mineral, Poland) as the reference electrode. The auxiliary electrode was a platinum wire (Mineral), and a carbon paste electrode (CPE) was used as the working electrode. All measurements were carried out in a voltammetric cell with a 15 mL volume. Optical microscopy images were recorded using Huvitz HRM-300 metallurgical microscope using PlanFlour EPI 10×/0.30 and 100×/0.90 objectives. Chromatographic analyses were performed using a 1220 Infinity LC system from Agilent equipped with a binary pump integrated with a degasser, an autosampler, a column oven, and a diode-array detector. All separations were achieved using a Zorbax C-18 (150 × 4.6 mm, 5 μm) analytical column (Agilent Technologies). Data acquisition and analysis were performed by applying OpenLAB CDS ChemStation Edition software. The pH was measured using a Hanna Instrument HI 221 pH-meter (Loveland, USA). For oil sample extraction and centrifugation vortex Multi Reax from Heidolph and centrifuge Mikro 200R from Hetich were used. Water was purified using either a Millipore Milli-QRG system (Millipore, Austria) or Polwater DL-3 (Polwater, Poland) in chromatographic and voltammetric studies, respectively.

Chemicals and reagents

The chemicals used in this study were of analytical quality. A standard stock solution of thymoquinone was stored at 4 °C and prepared by dissolving an appropriate amount of the compound in the distilled water (it has to be mentioned that such solution, is environmentally friendly and stable, but need 2 days for complete TQ dissolution)[38,39]. The stability of standard solutions and phytopharmaceuticals sample solutions during analysis was evaluated previously[40]. The investigations were based on leaving the solutions of TQ in tightly capped volumetric flasks, protected from light, on a laboratory bench and in a refrigerator. The solutions were stored for 3 h at room temperature or for at least 2 weeks under refrigeration at 2 °C. The obtained chromatographic results shown that no changes were observed for the tested solutions. The study confirmed the stability of TQ under tested conditions in the tested period of time[40]. Working solutions with lower concentrations were prepared by

appropriate dilution of the stock solution. The carbon paste used for the measurements consisted of graphite powder (Alfa Aesar, Germany) and paraffin oil (POCH, Poland) in a ratio of 1.0 g of graphite to 0.3 mL of oil. As the supporting electrolytes hydrochloric acid (pH range 0.3–1.4), and Britton-Robinson buffers (BR, pH 2.0–6.0) were used. Buffer components were purchased from Avantor (Poland). Ethyl alcohol absolute 99.8% was supplied by POCH (Gliwice, Poland). HPLC-grade methanol and acetonitrile were obtained from J.T. Baker (Deventer, Netherlands).

HPLC reference method

The reference HPLC analysis was performed at 25 °C on a reversed-phase analytical column. The mobile phase consisted of water 30% and acetonitrile 70%, and it was pumped at a flow rate of 1.0 mL·min^{-1}. Chromatographic separation was achieved in 3.8 min. The injection volume was 2 µL. The analyte was detected and quantified by UV absorbance at a wavelength of 254 nm. Identification of the TQ peak was based on a comparison of retention times and UV spectra, taken at the real-time analysis, with a corresponding set of data obtained for the authentic standard compound.

A series of experiments were designed to assess the linearity, accuracy, precision, LOD, and LOQ, to evaluate the HPLC-UV method for the determination of TQ in oil samples. A six-point calibration curve of TQ in the range of 6.1–73.2 mmol·L^{-1} oil was obtained using least-squares linear regression analysis of peak area vs. concentration of standard solution. The linear regression equation was $y = 126.17x + 25.825$ and showed good linearity with square correlation coefficients of 0.9944 (for verification of the TQ peak purity please see Fig. S8 and Fig. S9). Based on a signal-to-noise ratio of 3 and 10, the LOD and LOQ were 1.2 mmol·L^{-1} of oil and 3.0 mmol·L^{-1} of oil, respectively. Moreover, the precision and accuracy of the method was in the range 2.7–5.7% and 96.2–109.2%, respectively.

Analysis of oil extracts and dietary supplements

The effectiveness of the developed method was checked on oil extracts available on the market (*Pasieki Rodziny Sadowskich* and *Semco*) and extracts available from dietary supplements made from black cumin seed oils (*Laboratorium Biooil* and *Olvita*).

Extract preparation procedure for oil samples: 100 µL of oil was mixed with 800 µL of methanol and stirred for 10 min at 1850 rpm in darkness at room temperature. The samples were then centrifuged for 10 min at 12,000 rpm in darkness at 4 °C. The resulting supernatants were collected into fresh tubes.

Extract preparation procedure for oil samples in capsuled supplements: capsules made with gelatin, glycerin were open using a steeliness scalpel and 100 µL of oil was transferred by micropipette to 2.0 mL tube and mixed with 800 µL of methanol. The mixture was stirred for 10 min at 1850 rpm in darkness at room temperature and then centrifuged for 10 min at 12,000 rpm in darkness at 4 °C. The resulting supernatants were collected into fresh tubes.

In the SWV analysis, samples for measurements were prepared by taking 50 µL of the previously prepared extract and diluting it with methanol (99.8%, Baker Analyzed) to a final volume of 500 µL. The SWV analysis was carried out under optimized conditions. In the case of HPLC analysis, 80 µL of the methanolic extract was diluted with 720 µL of methanol in glass HPLC vials. The dilution was necessary due to the excessively high concentration of the analyte in the original sample.

Results and discussion

Voltammetric behaviour of thymoquinone on the carbon paste electrode

The preparation of the working electrode surface is a critical yet often underestimated step in achieving reliable and reproducible electrochemical measurements. The condition of the electrode surface directly influences the sensitivity, selectivity, and overall analytical performance of the electrochemical system. Proper surface preparation is essential to ensure consistent electron transfer kinetics and to minimize surface contamination or fouling, which could compromise the accuracy of the results. In this study, significant emphasis was placed on optimizing the carbon paste electrode surface preparation, recognizing its vital role in subsequent analytical determinations. Two distinct methods were evaluated to identify the most effective approach for refreshing the electrode surface. The first method involved polishing the electrode on a dry piece of filter paper until an even surface was achieved. In contrast, the second method employed the same polishing technique but utilized a wet filter paper instead. These methods were compared to determine which provided the most stable and reliable surface for thymoquinone determination, ensuring that the electrode surface was optimally prepared for precise and reproducible measurements. Randles-Sevcik eq.[31,41] was used to calculate electroactive surface area for electrodes refreshed with both methods (Table 1). As shown in Table 1 electrode refreshed on the dry filter paper had slightly smaller electroactive surface than electrode refreshed on the wet filter paper. However, refreshment procedure based on dry filter paper guaranteed very good repeatability, which is the most important factor in electroanalysis. Optical microscopy (OM) imaging was used to verify these conclusions. OM results collected on electrodes polished using wet and dry filter papers are shown in Fig. 1a and b, respectively. Main

Electroactive surface [cm^2]	
Dry filter paper	Wet filter paper
0.0545 ± 0.0002	0.0642 ± 0.0015

Table 1. Average CPE electroactive surface calculated after 5 measurement series.

figure and insets show images recorded using 10× and 100× magnifications, respectively. The straightforward difference between images is their shade but this might be result of automatic adjustment of contrast caused by their different reflectance (inversely proportional to smoothness). To better characterise samples, OM images using 100× magnifications (see insets in Fig. 1a and b) were also recorded. Their careful comparison shows that for electrode polished using wet filter paper some regions of the image remain out of focus, which indicate that roughness is larger than depth of field which for used objective is approx. 1 µm. This observation allows to conclude that wet filter paper polishing results in higher roughness of electrode surface. Increased electrode roughness due to the use of wet filter paper can have several implications for future measurements, particularly in terms of electrochemical response and reproducibility. A rougher electrode surface can enhance the effective surface area, potentially leading to higher current responses and improved sensitivity. However, this effect may not be entirely predictable or uniform, introducing variability between measurements and making it more challenging to achieve consistent results.The roughening of electrode surface can be simply explained by non-uniform dissolving of the electrode surface as a result of contact with wet filter paper. In consequence preferential abrasion of the samples is observed leading to formation of grooves observed using OM. Therefore, as OM imaging confirmed electrochemical observations, refreshment procedure based on dry filter paper was used in all further experiments. In our study, we prioritized the dry filter paper approach due to its simplicity, reproducibility, and minimal risk of altering the electrode surface composition through uncontrolled chemical or electrochemical reactions. While electropolishing is a well-established technique for smoothing and cleaning electrode surfaces, it often introduces additional variables, such as changes in surface roughness and oxide layer formation, which could influence the electrochemical response in ways that are difficult to standardize. Electrode roughness is generally influenced by multiple factors, and thus, standardized preparation methods are essential to ensure reproducibility. In this study, we used filter paper for surface cleaning, a procedure routinely employed in our laboratory in the case of paste electrodes, which has consistently yielded comparable results across multiple experimental series. The average relative standard deviation of peak current measurements were not exceeding 3 and 5% for dry and wet filter paper, respectively.

The next critical aspect of optimizing electrochemical measurements is the careful selection of the supporting electrolyte's pH, which plays a pivotal role in influencing the electrode reaction, as well as the kinetics and thermodynamics of the charge transfer process during the measurement. The pH of the electrolyte can significantly alter the electrochemical behaviour of the analyte, affecting not only the peak current but also the potential at which the redox process occurs[42,43]. To identify the most suitable electrolyte for thymoquinone analysis, an extensive series of voltammetric experiments was conducted across a variety of solutions, starting with the Britton-Robinson (BR) buffer, which allows for a broad pH range. Since the choice of supporting electrolyte is crucial in selecting optimal voltammetric conditions, initial studies aimed to maintain a constant blank solution composition, adjusting only the pH while considering acid-base equilibria. This initial screening revealed that thymoquinone exhibited its most pronounced electrochemical oxidation signals under strongly acidic conditions. Basic pH range of BR buffers was not investigated, as increasing the pH even to neutral resulted in a significant decrease in the recorded response. Given our assumption of classic hydroquinone-to-quinone conversion, extending the study to the entire pH range of BR buffers was deemed unnecessary. Recognizing the importance of maximizing signal intensity for analytical sensitivity, subsequent investigations were focused on hydrochloric acid (HCl) solutions, exploring a pH range from 0.3 to 1.6 (sample voltammograms are shown on Fig. 2).

The results indicated a clear pH-dependent variation in the electrochemical response, with the highest thymoquinone signals observed in HCl pH 0.7 (Fig. 2). Under such conditions cyclic voltammograms contains single signals both in anodic and cathodic branch (Fig. S1), showing electrochemical behaviour of quinones. Therefore, we assume that dihydrothymoquinone can accept one or more protons, leading to the formation of protonated dihydrothymoquinone and its conversion into a positively charged oxonium species. Such protonation increases electron deficiency, making the molecule more prone to oxidation. Since the potential scan starts from potential more negative than the reduction of thymoquinone and directs towards more positive potential it is favourable to oxidize dihydrothymoquinone back to thymoquinone. Comparing anodic to cathodic signal under CV conditions the ratio is 1.30, when potential scan is starting from negative potential. Under reversed potential direction scan the same ratio is equal to 1.25 (data not shown). Therefore, under such experimental conditions more favourable is to perform potential scan in the positive direction (peak potential separation in CV studies

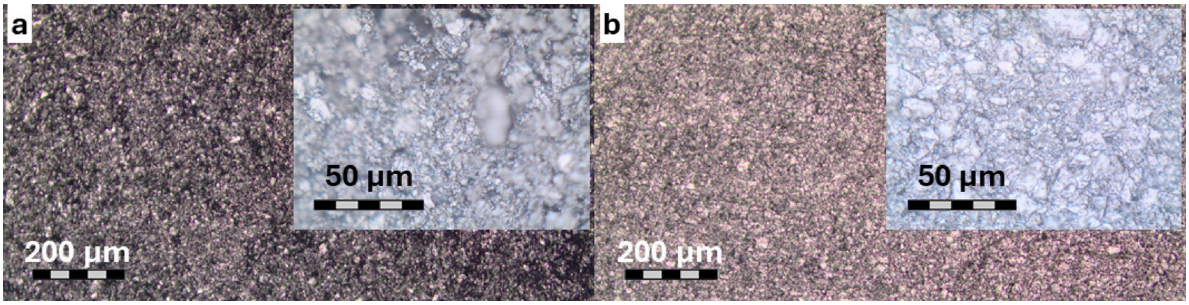

Fig. 1. Optical microscopy results recorded on samples polished using (**a**) wet and (**b**) dry filter paper. 10× magnification is shown in main part, while 100× in insets.

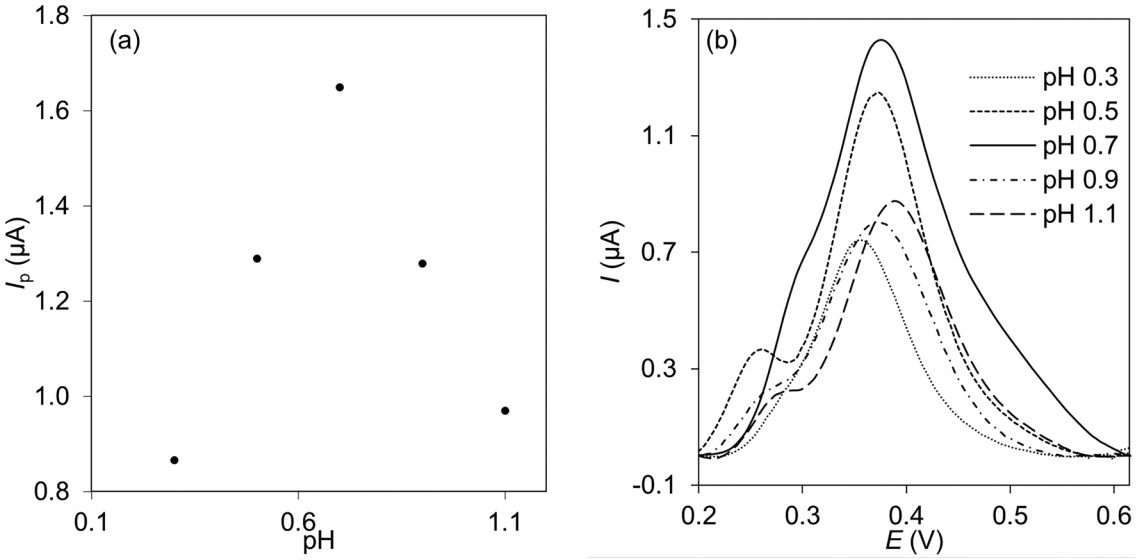

Fig. 2. (**a**) Dependence between 20 μmol·L^{-1} TQ SW peak current and pH of hydrochloride acid supporting electrolyte solution; (**b**) respective SW voltammograms. The SW experimental conditions were: frequency 50 Hz, amplitude 25 mV and step potential 2 mV.

with 2.0 μmol·L^{-1} TQ reached 171 mV). Consequently, a hydrochloric acid solution at pH 0.7 was selected as the optimal electrolyte for further electrochemical studies, providing a robust platform for subsequent analytical determinations.

The next stage of the research involved determining the optimal parameters for the SWV technique, including amplitude, frequency, and potential step. The first parameter tested was the frequency, which was varied in the range of 8–100 Hz. The TQ signal increased linearly up to a frequency of 50 Hz then the values changed non-linearly. Therefore, a frequency of 50 Hz was used for further studies. Another parameter investigated was the amplitude. The dependence of peak current shaped and height on amplitude was studied in the range of 10–80 mV. An increase in peak currents was observed over the entire range of amplitudes investigated. However, for amplitudes greater than 25 mV, the shape of the recorded signals deteriorated significantly. Due to this situation, an amplitude of 25 mV was used for further measurements. Influence of step potential was checked in range 1–25 mV. The peak current values increased up to step potential of 10 mV and then varied in a non-linear manner. Additionally, when determining the potential step, the only voltammogram with a good shape was at a potential step of 2 mV. A potential step of 2 mV was used for further studies. In conclusion, the following parameter values of the SWV technique were selected for further analysis: a frequency of 50 Hz, an amplitude of 25 mV, and a potential step of 2 mV.

Establishing an electroanalytical protocol for thymoquinone

After specifying the optimal parameters of the technique and determining the supporting electrolyte and pH, measurements of the TQ standard solution were made in the concentration range from 0.1 to 100.0 μmol·L^{-1}. During the measurements, it turned out that an overlapping signal appears with most of studied TQ contents (cf. Figure 3). A series of additional measurements with adjusting the conditions, e.g. adding surfactants or SW parameters adjusting in order to separate the peaks were performed (data not shown), which had no positive effect on peak transparency. The standard addition method is most often used to quantify analytes in complex real samples; however, its application is preceded by verifying the range, in which the analytical signal increases linearly with the analyte concentration increment. Although the recorded data seems to follow the typical voltammetric behaviour with concentration increment, it seemed that the complex signal shape may affect the chance for establishing broad range quantification of the analyte. To identify the most appropriate method for establishing the calibration range, we explored several approaches for analyzing the recorded voltammograms with complex electrochemical peak morphology, namely three methods were investigated to establish the calibration range. The first method involved using the highest recorded current within a given potential range. The second method focused on the specific area under the peak, which is related to the charge passed during the electrode reaction process. The third method defined the relationship between the sum of net currents recorded at each consecutive data point in the peak region (so called cumulative current) and the concentration of TQ, as defined by other methodologies[40]. It seems that each of the above-described methods of data interpretation in voltammograms and determination of calibration curves may give different conclusions regarding the linear relationship of $I = f(c)$. To assess whether and how the analysed, as described above, data affect the resulting analytical procedure, calibration curves, statistical parameters, etc., we used the same set of voltammetric records (sample voltammograms are shown on Fig. 3) for each calibration approach, which was then analysed using the three methods mentioned above.

Signal analysis	Linear range ($\mu mol \cdot L^{-1}$)	Slope ($\mu A \cdot L \cdot mol^{-1}$)	Intercept (μA)	R^2
Peak height	0.7–70.0	0.0428	0.5087	0.999
Peak area	0.1–1.0	0.0300	0.0555	0.996
	1.0–100.0	0.0054	0.0875	0.998
Cumulative current	0.1–1.0	0.0716	0.1650	0.999
	1.0–10.0	0.0068	0.2292	0.998
	10.0–100.0	0.0038	0.2558	0.997

Table 2. Statistical parameters of calibration curves based on different signal analysis.

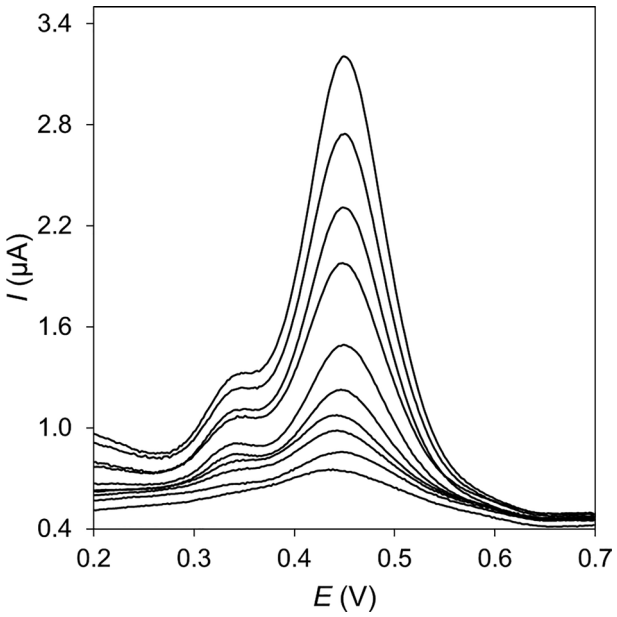

Fig. 3. SW voltammograms for various concentrations of TQ, $c_{(TQ)}$ = (0.7, 3.00, 5.00, 7.00, 10.00, 20.00, 30.00, 40.00, 50.00, 60.00 $\mu mol \cdot L^{-1}$); supporting electrolyte: HCl solution pH 0.7. The other experimental conditions were as Fig. 2.

As mentioned, the first approach was performed using the most known dependence between peak height (current) and analyte (thymoquinone) increasing content. This method consisted of determining the highest point of the signal peak on the voltammogram, which in this case was at a potential of approximately 0.45 V, and making out its height, which was then measured using the software used to control the potentiostat, i.e. the GPES software. In such way the linear range was determined in thymoquinone concentration range from 0.7 to 70 $mol \cdot L^{-1}$ (Fig. S2), while basic statistical parameters are given in Table 2.

Another approach used in the present study was the dependence between the peak area and the TQ concentration (method 2). The area was determined in the potential range from 0.28 to 0.65 V. For this, as in the previous methodology, data determined with the GPES software was used. Using this method, two linear ranges were identified. The first relationship ranges from 0.1 to 1.0, and the second from 1.0 to 100.0 $\mu mol \cdot L^{-1}$. Both calibration curves are shown in Fig. S3 and basic statistical parameters are given in Table 2.

The last method of defining the signal's dependence on concentration involves the summation of all measured current data points within the potential range of 0.28 to 0.65 V, which is in the same potential interval as for method 2. The hereby values obtained were assigned to the corresponding dihydrothymoquinone concentrations, and a linear dependence of cumulative current on the thymoquinone concentration was determined. The protocol was performed using the Microsoft Excel software after extraction of voltammogram raw data. In this approach (method 3) the concentration dependence had three linear ranges with TQ concentration increment: from 0.1 to 1.0, then from 1.0 to 10.0, and the last one from 10.0 to 100.0 $\mu mol \cdot L^{-1}$ (cf. Fig. S4 and Table 2).

Here it should be also mentioned that the sensitivity manifested through slopes of calibration curves is an important aspect of analysis. Method 1 is a relatively broad range dependence, but lower slope value is observed, probably due to the averaging the response over a broad range of measuring points. Compared with other methods, no 3 shows a significantly higher slope (167%) compared to method 1 in the lowest concentration range being the most important part of current concentration relationship. One should also keep in mind that the linear range in method 1 is narrowed (starting from 0.7 $\mu mol \cdot L^{-1}$ only) in the lowest concentration region, which is actually the most desirable one, while the other two data analysis approaches are dealing precisely within

those lowest applied concentrations of TQ in the solution. The observation of a linear dynamic range divided into two or three linear parts in voltammetric analysis, rather than a single broad range, can be attributed to several factors related to the electrochemical behavior of the analyte, the electrode surface, and the experimental conditions[44]. The key reasons for this phenomenon might stem from the fact that at low concentrations, the electrode surface may be fully active, allowing for a linear response. As the concentration increases, the active sites may become saturated, leading to a reduced rate of electron transfer, resulting in a non-linear response at higher concentrations[45–47]. Moreover, the order of the electrode reactions can vary with concentration, which affects the current response. Higher concentrations might shift the reaction order, changing how the current scales with concentration[48,49]. Another important aspect that should be taken into account is the fact that at higher concentrations, the products of the electrochemical reaction may begin to interfere with the reaction, causing changes in the voltammetric response and resulting in multiple linear regions[50]. One must keep in mind that the quinone-hydroquinone electrochemical systems often involve intermediates in a highly reactive radical form[30]. The observation of multiple linear regions in concentration dependence during voltammetric analysis reflects the complex interplay of electrochemical kinetics, mass transport, and electrode surface characteristics. Understanding these factors can provide further insights into optimizing the analytical method and improving the accuracy of concentration measurements. Here in this case, we can also observe how different approach in analysis of received data can lead to a simplified broad range concentration dependence, but, on the other hand, having no possibilities to observe subtle changes in the overall situation happening at the electrode surface. This is why an approach using a simple peak height is insensitive to slight changes in the overall perception of electrode mechanisms at the expense of limited linear concentration range[51]. Whereas other two ways of signal analysis have more strict relationships with origin roots stepping behind the observed scenario and therefore resulting in a broader concentration range relationships, which is being more acute on a given analyte concentration and finally limiting the applicative aspect of the particular approach based on signal peak area or summed cumulative current[52,53].

The limit of detection (LOD) and limit of quantification (LOQ) were determined based on signal-to-noise ratios, with the LOD defined at a ratio of 3:1 and the LOQ at 10:1, following standard procedures[54]. These values were calculated consistently for each of the methods applied. In method 1, the noise level was averaged from a blank sample at the peak potential, yielding LOD and LOQ values of 8.9 nmol·L^{-1} and 29.8 nmol·L^{-1}, respectively. For method 2, the limits were derived from the averaged noise surface area of the blank signal, resulting in values of 0.037 nmol·L^{-1} for LOD and 0.123 nmol·L^{-1} for LOQ. In method 3, a similar approach to method 1 was employed, but the cumulative background current was also taken into account, giving LOD and LOQ values of 15.8 nmol·L^{-1} and 52.8 nmol·L^{-1}, respectively. These results demonstrate that, despite the broader linear range offered by method 1, methods 2 and 3 provide significantly enhanced sensitivity, making them highly advantageous for trace-level detection in complex matrices, thus broadening the applicability of these techniques in precise quantitative analyses. Overall, these findings suggest that the combined use of different methods may offer a more comprehensive approach to analyte quantification, balancing sensitivity with a broader detection range, thus paving the way for more versatile applications in fields requiring precise trace analysis, such as pharmaceuticals or environmental monitoring.

Optimization of TQ extraction and chromatographic analysis from *Nigella sativa* oil

Sample preparation is a critical part of the analytical procedure, especially when quantifying TQ in *Nigella sativa* seed oil. Liquid–liquid extraction was applied to extract TQ from the oil, and several key parameters were optimized to improve yield, including the type and volume of the organic solvent, extraction time, and the method used (vortex or ultrasonic bath). Among the tested organic solvents (methanol, ethanol, acetonitrile), methanol was selected for further experiments due to its cost–effectiveness and previously documented use in TQ extraction methods[40]. Furthermore, it was observed that centrifugation after extraction improves the separation of the organic fraction from the oil. Subsequent optimization steps focused on determining the optimal volume of methanol and the required time for effective TQ extraction. It was found that higher sample dilutions (80–fold or more) increased extraction efficiency, and for future experiments, a methanol volume of 800 µL with a 10–fold dilution was chosen. Additionally, 10 min of vortexing at 1850 rpm was found to be sufficient for complete extraction of TQ. A way of agitation of a sample during liquid – liquid extraction impacts the extraction efficiency. Different methods of shaking, such as, manual shaking or shaking using different types of equipment dedicated for sample mixing, with a changeable speed and temperature of agitation can be used. The possibility of manual shaking is usually rejected due to a low repeatability caused by the lack of control the intensity of shaking and the temperature at which the process is carried out. The yield of extraction depends on the speed of shaking. Our laboratory is equipped with thermoshaker with variable sample shaking intensity ranging from 100 to 1850 rpm. In this studies highest speed of shaking was used. Due to the very good solubility of TQ in methanol, the liquid – liquid extraction process was very short and repeatable. The impact of temperature on the yield of extraction is crucial, especially, if volatile solvents are used for extraction. The highest influence of temperature on the yield and precision of extraction process is usually observed in temperature over 40 °C. Evaporation of methanol during the extraction process causes an increasing of the concentration of an analyte in the extract and lowering repeatability of the process. Due to very good results obtained for accuracy and pression of the method the prosses of optimization of TQ extraction in the presented assay involved solvent type, volume, and extraction time optimization only. For more detailed data on solvent type, volume, and extraction time optimization, please refer to the Supplementary Information, where corresponding figures (Fig. S5–S7) provide a more in–depth visual representation of these parameters and their effects on yield and precision.

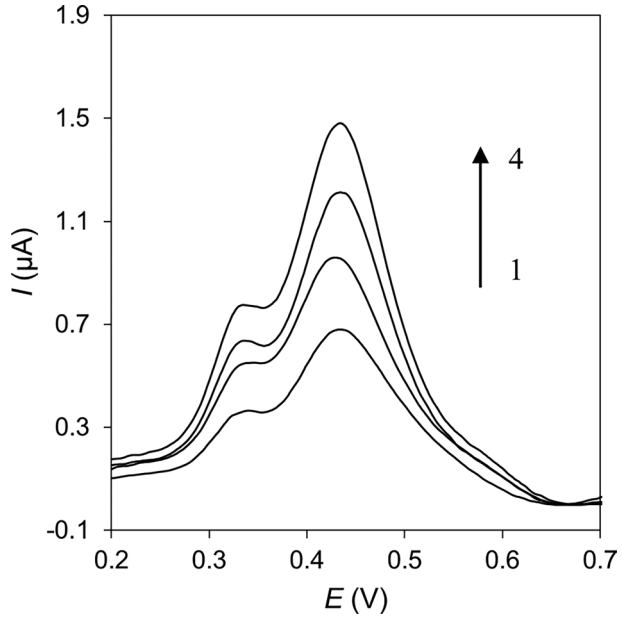

Fig. 4. SW voltammograms of TQ determination in oil sample (from Semco) using the standard addition method (1 – sample, 2–4 – standard additions); experimental conditions as in Fig. 3.

Sample	Detected in the sample (mg·mL^{-1})	HPLC reference method (mg·mL^{-1})	BIAS	E_1 [a] [%]
Black cumin seed oil				
Semco	5.77	5.73	0.04	0.70
Pasieki Rodziny Sadowskich	3.46	3.54	−0.08	2.26
Diet supplement				
Biooil	1.77	1.70	0.07	4.12
Olvita	3.64	3.60	0.04	1.11

Table 3. Determination of thymoquinone in real samples, ($n = 3$) [a] Relative error = [(SWV – HPLC value) / HPLC value] × 100.

Analysis of oil and dietary supplement

The determination of thymoquinone (TQ) in selected oil samples and dietary supplements was conducted to verify the analytical performance of the newly developed electroanalytical method. The method employed for preparing oil solutions is detailed in the "Analysis of Oil Extracts and Dietary Supplements" section. In these experiments, the standard concentration dependence of the peak current height, as measured by square-wave voltammetry (SWV), was utilized to determine the TQ concentration in the samples, adopting the standard addition method. Even though the other calibration curve construction methods offered lower detection limits, their use was not necessarily due to the high TQ content in the analyzed samples. Representative voltammograms obtained from selected oil samples are depicted in Fig. 4, which illustrates the clear and precise detection of TQ under optimal conditions. Importantly, to validate the accuracy and reliability of the developed SWV method, all results were cross verified using a conventional HPLC reference method. The comparative results from both methods are compiled in Table 3.

The *BIAS* values observed are relatively small, indicating a high degree of accuracy, reinforcing the reliability of our approach in practical applications. The comparison reveals a strong correlation between the outcomes of the SWV method and the HPLC reference method, indicating that the SWV approach with a carbon paste electrode is not only effective but also provides comparable results to the more established liquid chromatography techniques. This reinforces the robustness of the SWV method for practical applications in determining TQ content in various matrices, such as oils and dietary supplements.

In addition to demonstrating comparable accuracy, the SWV method offers several key advantages. The electrochemical approach is inherently more time-efficient and cost-effective than HPLC, requires fewer expensive reagents and less complex instrumentation just to name a few. These benefits make the SWV technique particularly attractive for routine analysis in both research and industrial settings, where quick and reliable quantification of TQ is required.

The successful application of the developed method, coupled with the strong agreement between SWV and HPLC results, supports the conclusion that the SWV-based approach with a carbon paste electrode is a viable alternative to liquid chromatography for the quantification of TQ. Furthermore, the simplicity, low cost,

and speed of the method make it a practical solution for regular use in laboratories, particularly when high throughput and cost efficiency are critical factors.

Conclusion

In conclusion, this work presents an optimized electrochemical method for the determination of thymoquinone (TQ) using square-wave voltammetry with a carbon paste electrode. Key to the method's success was the identification of dry cleaning as the most effective procedure for achieving a highly reproducible electrode surface. This finding was based on both experimental measurements and microscopic analysis, which showed that dry cleaning, despite the smaller reactive surface, yields much higher reproducibility compared to wet cleaning or polishing. The developed approach, while offering a promising and accessible method for electrochemical analysis, has certain limitations that must be acknowledged. One key factor is the potential variability introduced by differences in the composition and properties of the filter paper used during electrode preparation. Variations in fibre structure, porosity, and residual chemical additives in different brands or batches of filter paper could influence surface modifications, leading to inconsistencies in electrode roughness and wettability. These differences, in turn, could affect the electrochemical response, altering peak intensities, shifts in redox potentials, or changes in background currents. Similarly, the properties of the carbon paste used in electrode fabrication can impact reproducibility. Variations in particle size distribution, binder composition, and homogeneity of the paste can influence electron transfer kinetics and surface interactions with analytes. Even small inconsistencies in the mixing process or application method could result in slight deviations in electrochemical performance.

The optimization of experimental conditions was achieved through systematic testing, leading to the identification of ideal parameters: a 0.2 M HCl solution with a pH of 0.7 as the supporting electrolyte, a frequency of 50 Hz, an amplitude of 25 mV, and a potential step of 2 mV. These optimized conditions resulted in high sensitivity and selectivity, making the method robust for TQ analysis. Three methods for constructing calibration curves were evaluated, with the peak current height providing a broad linear range from 0.7 to 70.0 $\mu mol \cdot L^{-1}$, demonstrated to be highly suitable for practical applications. This method demonstrated limits of detection (LOD) and quantification (LOQ) of 8.9 and 29.8 $nmol \cdot L^{-1}$, respectively.

The developed electrochemical method was further validated through comparison with high-performance liquid chromatography (HPLC), which showed strong correlation between the two techniques. This confirms that the voltammetric method provides a reliable, fast, and cost-effective alternative to HPLC for the quantification of thymoquinone in Nigella sativa seed oil and dietary supplements.

Overall, the method offers significant advantages in terms of simplicity, efficiency, and cost-effectiveness, making it suitable for routine analysis. It holds great potential for broader applications in electrochemical sensing, particularly where rapid and precise quantification of bioactive compounds is required. The successful comparison with HPLC further reinforces its credibility as a viable analytical tool.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Received: 6 February 2025; Accepted: 20 May 2025
Published online: 29 May 2025

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Acknowledgements
S.S. would like to thank T.I. and T.S. for extremely valuable discussions during revision stage.

Author contributions
S.S., K.K., K.B., G.C., P.K. conceived and planned the experiments. M.ŚW., K.B., M.ŚL., K.K., D.G., S.S. carried out the experiments. K.K., K.B., S.S., M.ŚW. contributed to sample preparation. S.S., K.K., K.B., G.C., P.K., D.G. contributed to the interpretation of the results. S.S., K.K., K.B., D.G., M.ŚW., P.K. wrote the manuscript with input from all authors. All authors reviewed the manuscript.

Declarations

Competing interests
The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Smarzewska Sylwia has patent #p.448960, Sposób oznaczania 2-metylo-5-izopropylocykloheks-2,5-dien-1,4-dionu w suplementach diety i olejach spożywczych, (patent co-authors: Kamila Koszelska, Kamila Borowczyk, Dariusz Guziejewski, Michał Świderski, Łukasz Bogdański) pending to licensee. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-03347-3.

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