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Extraction-free colorimetric determination of thymol and carvacrol isomers in essential oils by pH-dependent formation of gold nanoparticles

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Abstract

An extraction-free method is described for the colorimetric determination of thymol (TY) and carvacrol (CA) isomers in essential oils by making use of the pH-dependent formation of gold nanoparticles (AuNPs). In solutions of pH 12, TY and CA form gold nanoparticles, while at pH ≤ 11 only CA does so. By taking advantage of this finding, two different approaches based on colorimetric assay (absorption at 550 nm) were developed: one at pH 12 for the determination of total CA and TY, and other at pH 9 and pH 12 for differential quantification of TY and CA. The former agrees with the well-established Folin-Ciocalteu method, and the latter provides a simple way for calculation of TY/CA ratio. The linear ranges are from 100 to 1000 μM at pH 9, and from 50 to 200 μM at pH 12. The limits of detection are 0.09 μM at pH 9, and 0.02 μM at pH 12. These features make this method simple, fast and reliable. Conceivably, it can be used to assess the quality of essential oils and may become a valuable alternative to more sophisticated, laborious and high time-consuming methods.

Keywords Gold nanoparticles · Cyclic voltammetry · Oregano · Thyme · Surface plasmon resonance · Folin-Ciocalteu

Abbreviations

TY	Thymol
CA	Carvacrol
AuNPs	Gold nanoparticles

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Introduction

Carvacrol (CA) and thymol (TY) are majority constituents of the essential oils extracted from oregano and thyme plants [1, 2]. Both are monoterpenes and isomers with an electroactive phenol group in this structure, so they are also termed phenolic compounds (see Fig. 1). They have attracted a great attention because of their antifungal, antibacterial, acaricide, and antioxidant properties [1, 3, 4]. Due to these properties, essential oils from oregano and thyme are proposed as natural antimicrobials and preservatives in agricultural and food applications [5–7]. Both compounds act synergistically and it was demonstrated that an adequate ratio between them caused a higher antimicrobial effect [8, 9]. Moreover, among the same subspecies, there are different chemotypes due to environmental, harvest time, extraction techniques and processing methods, causing compositional changes in the essential oils [10–13]. For these reasons, the total quantification of TY and CA, and even more interestingly, their proportion in essential oils, may be an excellent indicator of antimicrobial power of these natural products.

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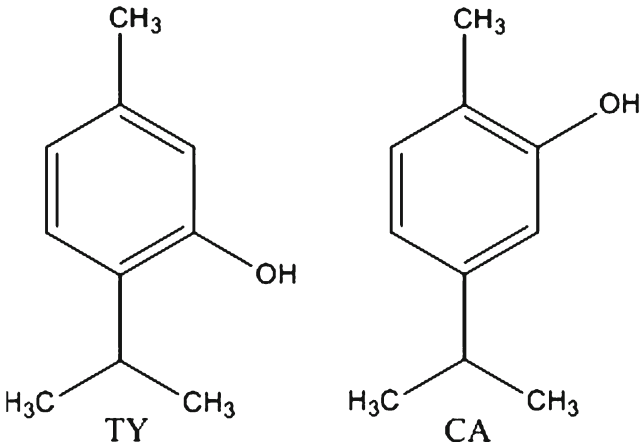

Fig. 1 Molecular structure of thymol (TY) and carvacrol (CA)

Common analytical methods employed for individually determination of thymol and carvacrol rely on separation techniques such as HPLC and GC [4, 14]. For example, both compounds were determinate by HPLC coupled to fluorescence [15], and UV-visible detection [16]; and by GC coupled to FID [17] and MS detection [10, 18]. These techniques possess a high-resolution power and sensitivity, which allow detecting even the minor components. These features are not necessary for the evaluation/characterization of essential oils components, considering the facts that TY and CA are very concentrated and the main components are responsible of their antioxidant and/or antimicrobial properties. Moreover, they are expensive and they are not present in all analytical laboratories. In consequence, simple and low-cost methods are sometimes preferred.

Another approach is the use of spectrophotometric methods for the determination of total phenolic content. Among them, Folin-Ciocalteu method is the most used in food and agricultural samples because it is simple and reproducible [14]. But, it is worth mentioning that this method overestimates the phenolic content in high extent which was pioneer demonstrated by Escarpa & González [19]. There are several examples about the measurement of total phenolic content in essential oil from thyme [20, 21] and oregano [22] by Folin-Ciocalteu method. Furthermore, electrochemical methods have been successfully employed for total phenolics [23] or class-phenolic determination [24], even for total CA and TY on single-walled-based screen-printed electrodes [25]. However, individual quantification of phenolics is not possible because their electrochemical responses overlap. Tonello et al. overcame this problem by using chemometric tools [26]. In this work, thymol, carvacrol and eugenol were quantified in honey samples by square wave voltammetry in combination with a multivariate calibration model using artificial neural networks [26].

On the other hand, the process of gold nanoparticles (AuNPs) formation is a valuable tool for an assessment of the antioxidant capacity in complex samples [27]. Basically, it relies on exploiting the antioxidant properties of phenolic compounds to reduce Au(III) to Au(0), generating gold nanoparticles. The amount of synthesized AuNPs is measured by absorbance around 550 nm due to surface plasmon resonance of AuNPs (colorimetric assay) [28–31]. This approach has shown excellent correlation with the Folin-Ciocalteu method in such a way that it has been proposed as alternative for total polyphenols determination [28, 29, 32]. It is interesting to highlight that it works even in fat-rich samples such as olive oil and chocolate [32, 33].

In this work, TY and CA capacity for AuNPs formation was evaluated from pH 8 to pH 12. Interestingly, at pH ≤ 11 only CA forms gold nanoparticles and at pH 12 both compounds. By taking advantage of this finding, two different approaches based on colorimetric assay (absorption at 550 nm) were developed. One at pH 12 for total CA and TY determination, and other at pH 9 and pH 12 for differential quantification of TY and CA isomers. The former agrees with the well-established Folin Ciocalteu method and the latter provides a simple way for calculation of TY/CA ratio. These approaches were successfully applied in essential oil samples without extraction protocols.

Materials and methods

Reagents

All reagents and solvents were of analytical grade and used without further purification. Thymol (TY), carvacrol (CA), Folin–Ciocalteu reagent, cetyltrimethylammonium chloride (CTAC, 25% in water), hydrogen tetrachloroaurate ($HAuCl_4.3H_2O$, 99.9%), sodium citrate ($C_6H_5Na_3O_7.2H_2O$), sodium phosphate monobasic monohydrate and sodium phosphate dibasic were obtained from Sigma Aldrich (Darmstadt, Germany, www.sigmaaldrich.com). Standard stock solutions of TY and CA (1×10^{-2} mol L^{-1}) were prepared in methanol (Panreac, Barcelona, Spain, www.itwreagents.com). Working solutions were daily prepared by accurate serial dilution from the stock solution of TY and CA using buffer. Stock solutions of CTAC (0.1 M), sodium citrate (0.3 mM) and hydrogen tetrachloroaurate (50 mM) were prepared in water. Essential oils (*Thymus vulgaris* and *Oreganum vulgare*) were purchased from local shops (Alcalá de Henares, Spain).

All solutions were prepared in Milli-Q water (Merck Millipore, Darmstadt, Germany, www.merckmillipore.com).

Apparatus

Absorbance measurements for colorimetric assay were performed by a Perkin Elmer spectrophotometer, diode array Lambda 35 (Waltham, MA, USA, www.perkinelmer.com). Cyclic voltammograms were carried out using an AUTOLAB PGSTAT 12 potentiostat/

galvanostat, run with GPES software, version (Eco Chemie, Utrech, The Netherlands, www.metrohm-autolab.com). The working electrode was a carbon screen-printed electrode (SPE) (DRP-110, Dropsens, Oviedo, Spain, www.dropsens.com) that includes a silver pseudo reference electrode and a carbon counter electrode. Transmission electron microscopy (TEM) images were taken with a Zeiss EM10C microscope (Oberkochen, Germany, www.zeiss.com).

Procedures

Gold nanoparticle-based method was adapted from *Scampicchio* et al. work [30]. Typically, a solution of 3.7×10^{-3} mol L^{-1} CTAC, 1.0×10^{-3} mol L^{-1} $HAuCl_4$ and 2.0×10^{-4} mol L^{-1} sodium citrate was prepared in a 1.0×10^{-2} mol L^{-1} phosphate buffer at different pHs (*reacting solution*) for each analysis. This solution was prepared adding the adequate volume of stock solutions into a vial containing phosphate buffer in the following order: (1) CTAC, (2) hydrogen tetrachloroaurate, (3) sodium citrate. The order is essential. Then, the adequate volume of working solution of TY or CA was added to the *reacting solution*, obtaining the desired TY or CA concentration. The final volume of these solutions was always 500 μL. Then they were stirred in a pulsing vortex mixer for 2 min. These solutions were heated at 45 °C for 10 min in a water bath. Finally, the absorption of these solutions were measured in a spectrophotometer from 200 to 800 nm. The UV-Vis spectroscopy showed a plasmon resonance surface band around 550 nm. The absorbance intensity was measured at the band maximum (550 nm).

Essential oils were analyzed in the same way but taking a known volume of them instead of TY and CA working solutions. In the protocol using a pH 9 solution, a known volume of essential oils (5 μL for *Thymus vulgaris* and 0.3 for *Oreganum vulgare*) were diluted in methanol (final volume 500 μL). 80 μL of these solutions were mixed with 420 μL of *reacting solution.* In the protocol using a pH 12 solution, a known volume of essential oils (0.4 μL for *Thymus vulgaris* and 0.3 for *Oreganum vulgare*) were diluted in methanol (final volume 500 μL). 30 μL of these solutions were mixed with 470 μL of *reacting solution.*

Cyclic voltammograms of thymol and carvacrol solutions in phosphate buffer were recorded from −0.2 to +0.4 V for pH 12 and from −0.2 to +0.8 V for pH 9. Scan rate of 50 mV/s was used.

In the Folin-Ciocalteu method, CA was used as standard. The protocol was as follows. 0.5 mL of standard solution, blank or essential oil were poured into a flask. Then, 0.5 mL of Folin-Ciocalteu reagent was added and this mixture was stirred for 3 min. Later, 10 ml of 75 g L^{-1} anhydrous sodium carbonate solution was added and, then, Milli-Q water up to a final volume of 25 mL. The mixture was stirred for one hour. Finally, the solutions were measured spectrophotometrically at 750 nm.

Results and discussion

Evaluation of carvacrol and thymol ability to form gold nanoparticle

Firstly, the ability of CA and TY to form gold nanoparticles was studied from pH 8.0 to 12.0 (see Fig. 2) to find the maximum absorbance signal. Unexpectedly, at pH ≤ 11, UV-vis spectroscopy reveal the surface plasmon band of AuNPs (around 550 nm) only for CA and not for TY. However, at pH = 12 both analytes show absorbance signals at 550 nm. To our best knowledge, this phenomenon was not previously described for these compounds.

To deepen in this finding, a comparative study was carried out between pH 9 and pH 12 for both compounds. AuNPs absorption spectra, colored solution photos and TEM images were recorded for TY, CA and the blank at pH 9 (see Fig. 3) and pH 12 (see Fig. 4). As it is shown in Fig. 3a, at pH 9 there is an absorption band around 550 nm for CA (red solution) but not for TY (colorless solution) both comparing with blank solution (colorless solution). Furthermore, TEM images (see Fig. 3c–e) confirm the presence of gold nanoparticles in CA solution and not for TY. This means that only CA is able to form gold nanoparticles at pH 9.

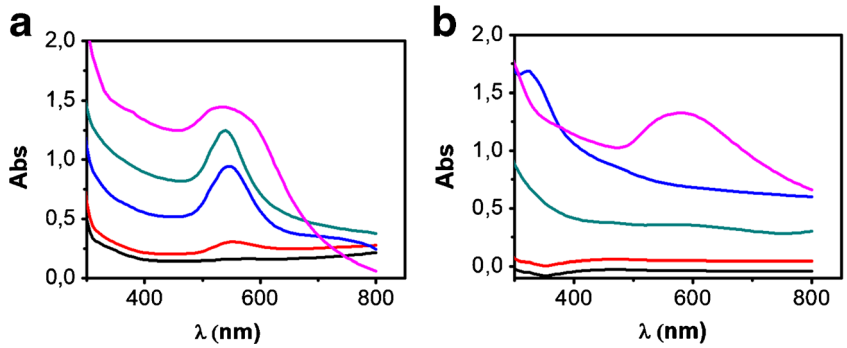

Fig. 2 UV-vis absorbance spectra of 0.25 mM carvacrol (**a**) and thymol (**b**) solutions at pH 8 (black line), pH 9 (red line), pH 10 (blue line), pH 11 (green line) and pH 12 (pink line)

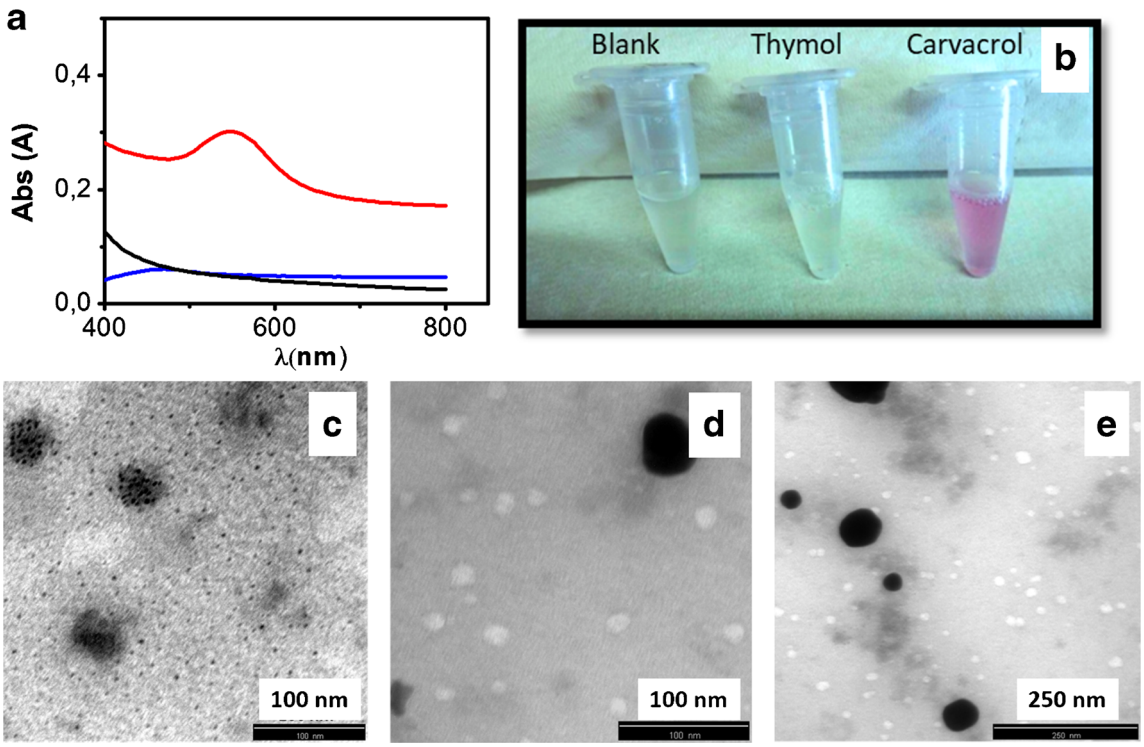

Fig. 3 Gold nanoparticle ability of TY, CA and blank at pH 9. **a** Absorbance spectra from blank (black line), TY (blue line) and CA (red line). **b** Photos from solutions after applying the gold nanoparticle synthesis protocol. **c**–**e** TEM images from CA (**c**), TY (**d**) and blank (**e**)

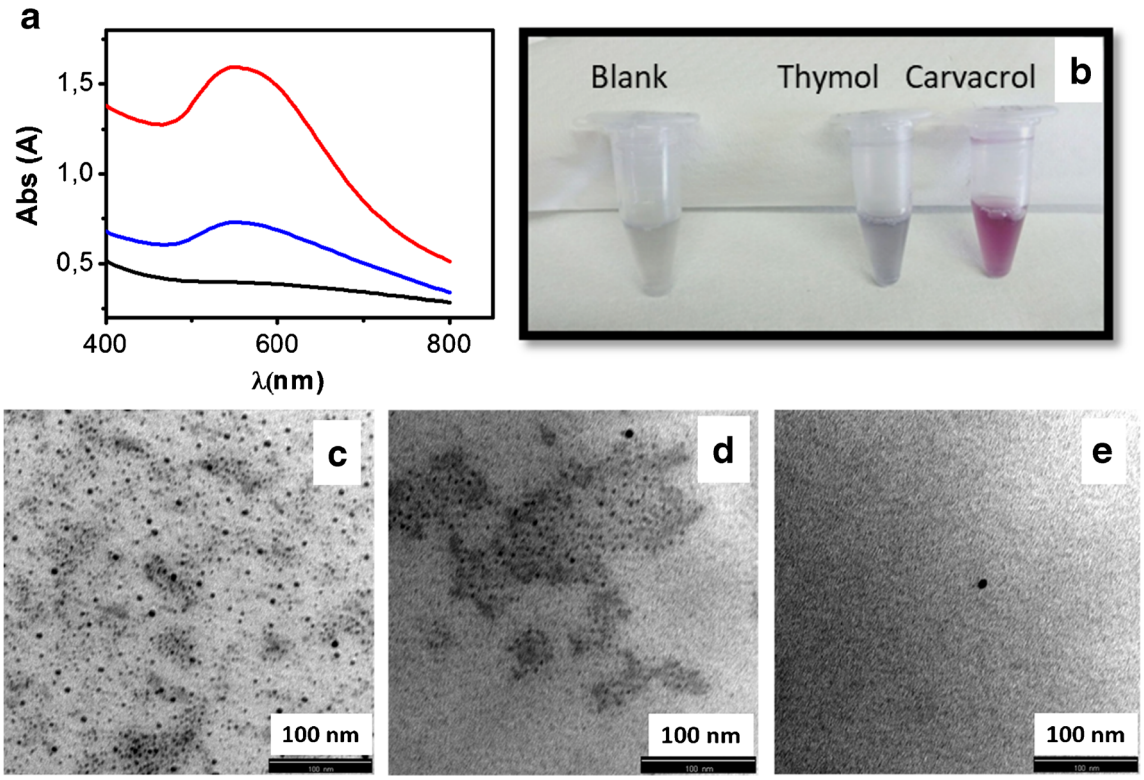

Fig. 4 Gold nanoparticle ability of TY, CA and blank at pH 12. **a** Absorbance spectra from blank (black line), TY (blue line) and CA (red line). **b** Photos from solutions after applying the gold nanoparticle synthesis protocol. **c**–**e** TEM images from CA (**c**), TY (**d**) and blank (**e**)

However, at pH 12 there are broad absorption bands around 550 nm with a long tail towards red region for CA and TY (see Fig. 4a). In addition, there is a small shift of the maximum absorption to red region for CA in comparison to pH 9. For these reasons, CA solution is purple and TY is dark blue as it is shown in Fig. 4b. We hypothesized this color change is related with the aggregation of gold nanoparticles and not for an increase in gold nanoparticle size because the size is similar between pH 9 and pH 12 ($\phi \leq 20$ nm). It is well-known that a decrease in the interparticle distance leads to a strong overlap between the plasmon fields of the nearby particles, causing a redshift in the surface plasmon resonance band [34, 35]. Finally, TEM images (see Fig. 4c–e) confirmed the presence of gold nanoparticles in CA and TY solutions and not for the blank. Therefore, CA and TY are able to produce gold nanoparticles at pH 12.

For the sake of a better understanding, cyclic voltammograms of TY and CA in pH 9 and pH 12 phosphate buffers were performed to find out if pH has a different effect on the electrochemical properties of these analytes. Figure 5 shows that the dependence of the electrochemical behavior on pH was similar for both compounds. The results show similar values of anodic peak potential for both compounds, and similar anodic peak potential shift toward less positive values with increased pH. This indicates that the oxidation is eased at high pH. Phenolic compounds have a weakly acidic character (the estimated values of pKa in aqueous solutions for TY and CA were pKa = 11.11 and pKa = 11.02 respectively). An increase of pH would allow the withdrawal of a proton from the phenolic molecule, helping to lower the overpotential for the oxidation reaction. Therefore, peak potentials do not explain the different behavior of CA and TY. Respect to the faradaic currents, CA showed higher values than TY, so it means CA possess higher antioxidant capacity than TY since both compounds exhibit identical oxidation potential. This can explain the differential pH behavior of both compound for gold nanoparticle formation.

Another possible explanation may be that the voluminous isopropyl group, present at ortho position respect to hydroxyl group in TY, hinders the formation/growing of gold nanoparticle but not the reduction of Au (III). This steric effect is however absent in CA because isopropyl group is at meta position respect hydroxyl group. Our results are not conclusive and we believe that more experimentation may be needed to support any conclusion.

Analytical routes for total CA and TY determination and for differential quantification of CA and TY isomers in essential oils

Based on the aforementioned results, it is possible to develop two analytical routes: one, to quantify only CA working at pH 9 and, another, to determine the total CA and TY concentration at pH 12. Therefore, calibration plots were recorded at pH 9 and 12 using CA as standard (see Fig. 6). An excellent linear correlation coefficient was obtained from 100 to 1000 μM at pH 9 ($r = 0.993$) and from 50 to 200 μM at pH 12 ($r = 0.994$). At pH 9, the calibration slope was 0.73 ± 0.03 μM^{-1} and the intercept 0.13 ± 0.02. The limit of detection was 0.09 μM

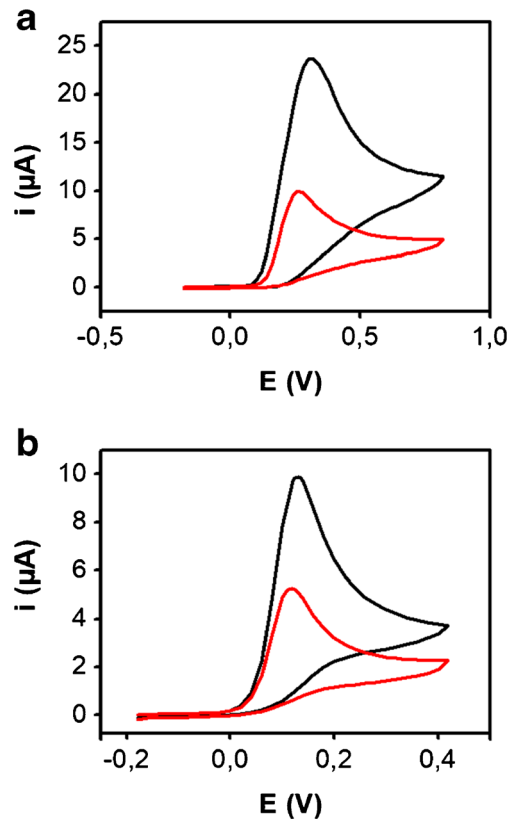

Fig. 5 Cyclic voltammograms of CA (black line) and TY (red line). **a** Analytes at 250 μM concentration dissolved in phosphate buffer pH 9, **b** Analytes at 100 μM concentration dissolved in phosphate buffer pH 12. Scan rate: 50 mV/s

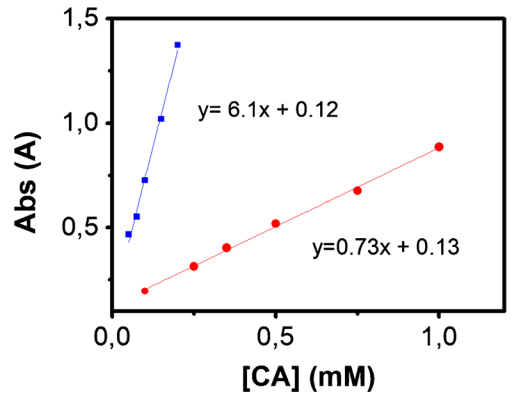

Fig. 6 Calibration plots for carvacrol using the analytical routes at pH 9 (red color) and pH 12 (blue color). Absorbance signals were measured at 550 nm

Table 1 Total phenolic determination in essential oils from Thyme and Oregano

Sample	Method	Concentration (%*w*/w) ± s	RSD (%) ($n = 3$)
Thymus vulgaris	AuNPs-based at pH = 12	0.46 ± 0.02	5
	Folin-Ciocalteu	0.44 ± 0.01	3
Origanum vulgare	AuNPs-based at pH = 12	0.75 ± 0.02	4
	Folin-Ciocalteu	0.70 ± 0.04	6

(3 S/N criterion using intercept standard deviation). At pH 12, the calibration slope was 6.1 ± 0.3 μM^{-1} and the intercept 0.12 ± 0.04 ($n = 3$). The limit of detection was 0.02 μM.

Both routes were applied to analyze oregano and thyme essential oils. Firstly, gold nanoparticle-based method at pH 12 was used for total TY and CA determination. In order to evaluate the validity of our method, the oregano and thyme essential oils were also analyzed by Folin-Ciocalteu method. Data are summarized in Table 1. The results show good reproducibility, and unexpected but interestingly good agreement with Folin-Ciocalteu method ($\alpha = 0.05$).

Then, the possibility of quantifying the amount of TY and CA isomers in essential oils by the pH-dependence of TY to form gold nanoparticles was also explored. Starting from the fact that the main components of the studied essential oils are TY and CA [2, 21, 22], we made the assumption that all AuNPs were exclusively formed by TY and CA. Next, thymus and oregano essential oils were analyzed by our method at pH 9 and pH 12 (see Table 2). At pH 12, CA and TY are quantified, and at pH 9 only CA is quantified, so subtracting the quantification values obtained at both pH, the amount of TY is estimated. Our results indicate that *Origanum vulgare* essential oil contains more CA than TY unlike *Thymus vulgaris* one, which contains mainly TY. These results are not confirmed with other methodologies but they are highly consistent with the bibliography [2, 3, 10, 11]. This route allows calculating CA / TY ratios and may be an interesting approach for measurement antimicrobial power of thyme and oregano essential oils.

Moreover, it is important to empathize that there is no need of any extraction procedure before analysis.

Finally, it is worth mentioning that these new methods work well just for the selected samples because TY and CA are the main components able to form AuNPs because of their antioxidant properties.

Conclusions

It has been demonstrated that TY and CA possess a different pH dependence with respect to the ability to AuNPs formation. By taking advantage of this finding, two different analytical routes were developed based on the measurement of AuNPs formation by absorbance at 550 nm. First route provides total CA and TY determination (at pH 12). Second route provides the individual quantification of CA and TY isomers by differential measurement of AuNPs formation between pH 12 and pH 9. A proof of concept was shown, applying these methodologies to thyme and oregano essential oils. The second route is a simple and fast way to characterize thyme and oregano essential oils based on CA / TY ratios, which may be a good indicator of the antimicrobial power of these natural products.

Moreover, it is worth mentioning that there is no need of any extraction procedure before analysis and that this approach works in fat-rich samples such as essential oils. In conclusion, AuNPs-based approach is a valuable alternative to other more sophisticated, laborious and high time-consuming ones for essential oils characterization.

Although these methodologies can be only applied to samples with CA and TY as main antioxidant components, this work paves the way to find other phenolic compounds with different pH-dependent ability to form AuNPs. These potential findings would allow designing new detection routes for selective determination of phenolics in essential oils and other natural products.

Table 2 Differential quantification of thymol and carvacrol in essential oils

Sample	Quantification at pH 12 (CA + TY) (%w/w)	Quantification at pH 9 (CA) (%w/w)	Quantification by subtracting (TY) (%w/w)	CA / TY ratio
Thymus vulgaris	0.46	0.10	0.36	0.3
Origanum vulgare	0.75	0.41	0.34	1.2

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Compliance with ethical standards The author(s) declare that they have no competing interests.

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