Multivariate approach in voltammetric identification and simultaneous determination of eugenol, carvacrol, and thymol on boron-doped diamond electrode

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Abstract

The paper presents multivariate partial least-squares calibration model defined for the identification and simultaneous determination of phenolic terpenoids—eugenol, carvacrol, and thymol—by means of the differential pulse voltammetry. For the first time, using boron-doped diamond electrode signal, electrochemical distinction of carvacrol and thymol, despite the short distance between the peaks (15 mV), was described. The analytes' concentrations applied in the calibration model were selected using the 3-factorial central composite design. The multivariate partial least-squares model, with optimized data pre-processing, was used for determination of eugenol, carvacrol, and thymol in the concentration ranges of 0.77–13.86, 0.58–10.40, and 0.73–7.32 mg/dm³, respectively. The quality of the method, expressed by the root-mean-squared error of calibration was 0.55, 0.20, and 0.26 mg/dm³ for the specified analytes. The recovery in the analysis of the pure signals was 98.92%, 101.62%, and 101.46%, what confirmed the possibility of clearly distinguishing the thymol and carvacrol peaks. In the spiked honey samples, the accuracy of eugenol, carvacrol, and thymol determination, expressed by the root-mean-squared error of prediction, was in the range 0.20–0.83 mg/dm³, and recovery in the range 80.9–111.9%. Application of the presented model not only enables electrochemical identification and simultaneous determination of eugenol, carvacrol, and thymol, but also allows rapid, accurate, and low-cost verification of authenticity of the commercial products.

Graphical abstract

Keywords Chemometrics · Electroanalysis · Terpenoids · Voltammetry

Introduction

Phenolic terpenoids: eugenol, carvacrol, and thymol

Terpenes, along with their derivatives called terpenoids, form a very large group of chemical compounds obtained mainly from plants. Up to now, more than 55,000 substances of this type have been discovered [1–3]. Due to their numerous and varied health properties, terpenes with terpenoids are of interest of many scientists. Among the

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terpenic derivatives, phenolic terpenoids such as eugenol, carvacrol, and thymol have found numerous applications.

Eugenol [2-methoxy-4-(prop-2-en-1-yl)phenol, CAS number: 97-53-0] is a phenolic terpenoid present in the form of a colorless or slightly amber liquid (Fig. 1) obtained from essential oil of clove (*Syzygium aromaticum*) which contains 73.6–84.1% of eugenol [1, 4–6]. Essential oils with lower content of eugenol are cinnamon oil (*Cinnamomum altissimum*, *Cinnamomum verum*, or *Cinnamomum cassia*)—1.2–14.7% [6–10] and laurel oil (*Laurus nobilis*)—1.4–2.2% [11]. These oils rich in eugenol are used in dietary supplements, cosmetics, aromatherapy, and phytotherapy, due to their anesthetic, antifungal, and insecticidal properties [7–15].

Carvacrol [2-methyl-5-(propan-2-yl)phenol, cymophenol, isothymol, CAS number: 499-75-2] is a phenolic terpenoid, geometric isomer of thymol, present in the form of yellowish liquid (Fig. 1) obtained from essential oils of dictamnus (*Origanum dictamnus*), oregano (*Origanum vulgare*), and thyme (*Thymus vulgaris*): the oil contains 42.9–82.3%, 8.6–65.1%, and 2.2–81.5% of carvacrol, respectively [5, 16–27]. Other oils rich in carvacrol are obtained from marjoram (*Origanum majorana*), lemon thyme (*Thymus pulegioides*), and summer savory (*Satureja hortensis*); its content in oil is: 2.2–48.7%, 32.8–62.6%, and 34.4–50.6%, respectively [5, 22, 23, 28–31]. Oregano and thyme essential oils are used in dietary supplements, aromatherapy, and phytotherapy for the treatment of colds, and as active antifungal, insecticidal, and antibacterial agents [5, 23, 25, 27, 29].

Thymol [5-methyl-2-(propan-2-yl)phenol, CAS number: 89-83-8] is a phenolic terpenoid, geometric isomer of carvacrol, present in the form of translucent crystals (Fig. 1) obtained from essential oil of thyme (*Thymus vulgaris*): the oil contains 14.4–63.0% of thymol [4, 9, 26, 27, 32]. Essential oils with lower content of thymol are oregano oil (*Origanum vulgare*)—2.9–7.43% [18, 19] and ajowan caraway oil (*Carum copticum*)—45.0–55.0% of the oil content. Essential oils rich in thymol—thyme and ajowan caraway—are used in dietary supplements, aromatherapy, and phytotherapy as expectorant, antiseptic, antispasmodic, antioxidant,

antifungal, and antimicrobial agents [29, 32–35]. Thymol itself is used in food, pharmaceutical, and cosmetic industries [32].

Due to their high biological activity products rich in these phenolic terpenoids have found wide application as: food or food additives, aromatic spices, para-pharmaceutical products, dietary supplements, herbal products, cosmetics, or fragrance raw materials [2, 7–15] and [29–37]. The lethal doses of these compounds are high—LD₅₀ are: > 2000, 810, and 980 mg/kg, for eugenol, carvacrol, and thymol, respectively (rat, orally) [38]; and what is important, in nature, these compounds are not present in such high concentrations.

Among the most recent literature reports (since 2015), the essential methods of determination have been developed for eugenol and thymol, while there are significantly fewer procedures for determining carvacrol (Table 1).

Currently, electrochemical methods of eugenol determination are dominant [12, 41, 42, 47]. Second large group used for this purpose is chromatographic methods, mostly coupled with MS [39, 40, 48, 49]. A similar situation is in the case of thymol, where numerous procedures have been developed using electrochemical methods [41, 46] and chromatographic methods coupled with MS [37, 43–45]. Determination of carvacrol is carried out mainly using chromatographic methods. A small number of electrochemical procedures for determination of carvacrol may result from too close position of the carvacrol and thymol peaks, preventing the proper analytical evaluation of the obtained results [50, 51].

Due to the frequent combined occurrence of carvacrol and thymol in a number of matrices of natural origin—such as essential oils, honeys, complex medicinal products, or cosmetics—and the ever-increasing demand for such products, it is important to develop cheap, fast, and effective methods for simultaneous determination of phenolic terpenoids. Currently, only procedures based on an expensive chromatographic separation are used for this purpose [44, 45]. Until now, voltammetric techniques have been used in work [41], where the authors employing square-wave voltammetry (SWV) and a glassy carbon electrode achieved the satisfactory results using artificial neural network (ANN)

Fig. 1 Structural formulas of the analyzed phenolic terpenoids

Table 1 Selected analytical methods used for determination of phenolic terpenoids

Phenolic terpenoid	Analytical technique	Linearity range, mg/dm ³	Limit of detection (LOD), mg/dm ³	Year of publication	References
Eugenol	GC–MS	0.001–0.05	0.0001	2015	[39]
	GC–MS/MS	0.005–0.500	0.001	2016	[40]
	SWV at GCE	^a	0.010	2016	[41]
	LSV at MIP/GN-CNTs-IL/GCE	0.821–3.284	0.016	2016	[42]
	DPV at GPE	0.049–8.210	0.014	2017	[12]
	DPV at BDDE	0.39–14.63	0.02	2019	This work
Carvacrol	HPLC–UV	1.0–80.0	0.8	2015	[43]
	SWV at GCE	^a	0.210	2016	[41]
	GC–MS	0.5–10.0	0.14	2017	[44]
	GC–MS/MS	0.002–0.500	^a	2018	[45]
	DPV at BDDE	0.29–11.56	0.02	2019	This work
Thymol	HPLC–UV	1.0–80.0	0.6	2015	[43]
	DPV at BDDE	0.586–15.022	0.586	2015	[46]
	HPLC–UV	0.5–15.0	0.05	2016	[37]
	SWV at GCE	^a	0.240	2016	[41]
	GC–MS	0.5–10.0	0.15	2017	[44]
	GC–MS/MS	0.002–0.500	^a	2018	[45]
	DPV at BDDE	0.34–9.52	0.02	2019	This work

^aNo data available

to define multivariate calibration model for eugenol, carvacrol, and thymol. In turn, in this work, the partial least-squares (PLS) algorithm was used, which enabled not only quantitative but also qualitative analysis. In addition, we achieved tenfold lower detection limits for carvacrol and thymol, operating on twice greater concentrations ranges of the considered analytes.

Therefore, further application of electrochemistry in combination with chemometric methods—especially multivariate analysis of data obtained experimentally—can be very promising way, allowing the extraction and visualization of analytical relationships that are not observed in classical analysis.

Design of the experiment using central composite design

One of the ways to optimize the number of experiments is the statistical design of the experiment (DOE). From numerous procedures of DOE [52, 53], in this work, the central composite design (CCD) was selected and constructed for three factors. The calibration samples (training set) contained constituent patterns: the full factorial points (values -1 or $+1$ on each axis), central point and its repetitions (value 0 on each axis), and star points (at some value $-\gamma$ or $+\gamma$ on each axis). Value of the parameter γ was calculated in [52] as follows:

$$\gamma = (2^k)^{1/4}, \quad (1)$$

where k is the number of factors—in this case, it is the number of used phenolic terpenoids, equal to three, and hence, $\gamma = 1.682$. CCD is inherently rotatable, which implies equal precision of estimation in all directions which depends only on the distance from the center of design space. Since the rotatability depends only on the value of γ , Eq. (1) is right [52, 53]. The central point was repeated in six independent experiments, and the total number of samples considered in training set was 20. For validation of the calibration model, the testing set was created containing variables equal to the half of the full factorial points levels (values -0.5 or $+0.5$ on each axis). The validation set was constructed of eight independent samples.

The correctness of calibration model

Multivariate PLS calibration is one of the basic techniques of supervised chemometric approach, which enables the simultaneous and precise determination of many substances, and elimination of the disturbing signal components, where each of the considered substances can be treated as an analyte or an interferent [54–57]. Due to the fact that PLS is the supervised method, this model is built using a calibration set of samples (training data set), and validated using one of the strategies, like cross validation by venetian blind with four data splits, which was applied in this work. Based on data from the training samples, the model predicts the values of samples from the external data set. Quality of the model and

correctness of this prediction is assessed by such parameters as: high correlation coefficient (r), low root-mean-squared error of calibration (RMSEC), low RMSECV, low root-mean-squared error of prediction (RMSEP), low mean relative error of prediction (REP), critical analysis of plots predicted= f (measured), and recovery test for the external samples [55, 56, 58, 59]. The coefficient r reflects the accuracy of matching [56], RMSEC, RMSECV, and RMSEP are calculated in [59] as follows:

$$\text{RMSEC} = \sqrt{\frac{\sum_{i=1}^I (y_i - \hat{y}_i)^2}{I - \text{LV} - 1}} \quad (2)$$

$$\text{RMSECV} = \sqrt{\frac{\sum_{i=1}^{I_t} (y_i - \hat{y}_i)^2}{I_t}} \quad (3)$$

$$\text{RMSEP} = \sqrt{\frac{\sum_{i=1}^{I_t} (y_i - \hat{y}_i)^2}{I_t}}, \quad (4)$$

where y_i is the reference value of the sample i , $\hat{y}_i$ is the model prediction for the sample i , I is the number of calibration samples, I_t is the number of samples left-out in the cross-validation process and I_t is the number of test samples for the validation set, and LV is the number of latent variables used in model. REP is expressed as follows [58]:

$$\text{REP} = \frac{\text{RMSEC}}{\bar{y}} \times 100\%, \quad (5)$$

where $\bar{y}$ is the average real concentration value for each analyte.

Results and discussion

Composition of the supporting electrolyte

At the beginning, composition of the supporting electrolyte and measurement parameters has been optimized in the typical way. The basic optimization criterion was to obtain the best separation of the individual analytes peaks and low, stable background level (baseline). While position of the eugenol peak did not require modification, the difference between the carvacrol and thymol peaks was problematically low [46, 50, 51]. In addition, the potential range was modified to avoid the additional effect of the supporting electrolyte degradation and baseline change, what could significantly hinder the definition of a multivariate model. Optimization of composition and pH value of the supporting electrolyte was made similar to [50]. A wide pH range—combined from 1.75 to 11.00—was considered through the use of four

buffers (Britton-Robinson, acetate, ammonium, and phosphate). 0.1 mol/dm³ acetate buffer pH 6.0 was chosen as the most optimal for the further research. In the acetate buffer environment, no significant effect of pH on the peak positions of the considered analytes was observed [50].

Effect of scan rate

Then, electrochemical behavior of eugenol, carvacrol, and thymol has been investigated by recording cyclic voltammograms (CV) using the scan rates of 0.05, 0.1, 0.2, 0.25, and 0.5 V/s (Fig. 2a–c).

Obtained CV signals suggest an irreversible electrode processes. Diagnostic criteria used to characterize an irreversible system are [60–63]: no reverse peak and linear relation between peak current (i_p) and square root of scan rate ($\nu^{1/2}$). The peak potential values should be proportional to the $\log(\nu)$ as the following Eq. (6) at 298 K:

$$\left| \frac{dE_p}{d \log(\nu)} \right| = 0.0296 / \alpha n_\alpha [\text{V}], \quad (6)$$

where E_p is the peak potential, α is a kinetic parameter that characterizes the effect of electrochemical potential on the activation energy of an electrochemical reaction and can be calculated according to the slope of E_p vs. $\log(\nu)$ in linear range; and n_α is the number of electrons involved in process. The last criterion of an irreversible system is given by Eq. (7) at 298 K:

$$E_p - E_{p/2} = 0.0477 / \alpha n_\alpha [\text{V}], \quad (7)$$

where $E_{p/2}$ is the potential where the current equals one half of the peak value.

In registered cyclic voltammograms, the anodic peaks of eugenol, carvacrol, and thymol are not accompanied by a cathodic peak (Fig. 2a–c), which indicates that their redox reaction is totally irreversible. In addition, the linear relationships between peak current (i_p) and the square root of scan rate ($\nu^{1/2}$) have been obtained (Fig. 2d and Table 2).

Further examination of CV at different potential scan rates (Fig. 2a–c) reveals that the peak potential shifts to more positive potentials on the increasing scan rate ν . The dependence of E_p on the $\log(\nu)$ for three considered analytes is shown in Fig. 2e and Table 2. Value of αn_α is 0.50, 0.66, and 0.40 for these three systems, what was obtained from Eq. (6). For the lowest of the considered scan rates, i.e., 0.05 V/s, according to Eq. (7), αn_α was also estimated—obtained values were in line with the previously given and were equal to 0.58, 0.69, and 0.49 for eugenol, carvacrol, and thymol, respectively. In the case of carvacrol and thymol, interpretation of the registered curves was hampered by the specific shape of the registered cyclic curves. Because, for irreversible processes, parameter α is in the range 0.3–1.0 [63], we can conclude that one electron participates in each

Fig. 2 Cyclic voltammograms by the scan rate of 0.05, 0.1, 0.2, 0.25, and 0.5 V/s obtained for: **a** 4.27 mg/dm³ of eugenol (navy blue), **b** 8.67 mg/dm³ of carvacrol (brick-red), and **c** 7.32 mg/dm³ of thymol

(green); in 0.1 mol/dm³ acetate buffer pH 6.0. Relation between anodic peak current i_p or peak potential E_p and scan rate ν : **d** i_p versus $\nu^{1/2}$, **e** E_p versus $\log(\nu)$, and **f** $\log(i_p)$ versus $\log(\nu)$

Table 2 Parameters of straight line fitted to cyclic voltammograms; where: i_p [μA]—peak current, ν —scan rate [V/s], E_p —peak potential [V], a—slope, b—intercept, and r—correlation coefficient

Phenolic terpenoid		Relation	a	b	r
Type	Concentration, mg/dm ³				
Eugenol	4.27	i_p versus $\nu^{1/2}$	3.03	0.06	0.9999
		E_p versus $\log(\nu)$	0.059	0.809	0.9978
		$\log(i_p)$ versus $\log(\nu)$	0.47	0.48	0.9998
Carvacrol	8.67	i_p versus $\nu^{1/2}$	4.97	−0.35	0.9961
		E_p versus $\log(\nu)$	0.045	1.10	0.9939
		$\log(i_p)$ versus $\log(\nu)$	0.65	0.72	0.9936
Thymol	7.32	i_p versus $\nu^{1/2}$	1.83	0.39	0.9933
		E_p versus $\log(\nu)$	0.074	1.09	0.9949
		$\log(i_p)$ versus $\log(\nu)$	0.34	0.33	0.9987

of the analyzed processes. It is compatible with the previous literature reports [41, 50].

In turn, dependence slopes of the straight line of the logarithm plots of peak currents vs. logarithm of scan rate (Fig. 2f and Table 2) were near the theoretical value of 0.5, what suggests that the electrochemical process is diffusion controlled [64].

Univariate calibration

Next, univariate calibrations were performed for each of the considered phenolic terpenoids (Table 3 and Fig. 3). From the recorded voltammograms, experimental backgrounds were subtracted by approximation, using polynomials of third degree.

The LOD value—calculated as three times the standard deviation of the blank (S_{blank}), divided by the slope (a) of the calibration curve—for each of the phenolic terpenoids was the same (0.02 mg/dm³). The obtained results show that sensitivity for thymol was higher than for eugenol and

Table 3 Parameters of the univariate calibration models for eugenol, carvacrol, and thymol (voltammograms in Figs. S1–S3), where S is standard deviation for the slope (S_a) and intercept (S_b). The experimental background was subtracted by approximation using third-degree polynomials

Phenolic terpenoid	Linearity range, mg/dm ³	E_p^* , mV	a , $\mu\text{A}/\text{mg}/\text{dm}^3$	S_a , $\mu\text{A}/\text{mg}/\text{dm}^3$	b , μA	S_b , μA	LOD, mg/dm ³	r
Eugenol	0.39–14.63	715	0.0863	0.0005	0.0319	0.0040	0.02	0.9996
Carvacrol	0.29–11.56	940	0.0708	0.0006	0.0491	0.0037	0.02	0.9992
Thymol	0.34–9.52	955	0.1056	0.0020	0.0556	0.0103	0.02	0.9970

*For 7.40 mg/dm³ of eugenol, 5.55 mg/dm³ of carvacrol, and 4.10 mg/dm³ of thymol – Fig. 3a

Fig. 3 **a** DP voltammograms of the analyzed phenolic terpenoids, obtained for 7.40 mg/dm³ of eugenol, 5.55 mg/dm³ of carvacrol, and 4.10 mg/dm³ of thymol in 0.1 mol/dm³ acetate buffer pH 6.0; without background subtraction (raw data). **b** Univariate calibration relation

for 0.39–14.63 mg/dm³ of eugenol. **c** Univariate calibration relation for 0.29–11.56 mg/dm³ of carvacrol. **d** Univariate calibration relation for 0.34–9.52 mg/dm³ of thymol

carvacrol—by 49% and 22%, respectively. The difference between the position of the carvacrol and thymol peaks was equal to ~15 mV (Fig. 3a). Such a small distance does not allow for identification and univariate calibration, if both analytes are present at the same time. The solution to this problem is to use the PLS calibration model.

Multivariate PLS approach

Subsequently, the training and validation curves for PLS multivariate calibration model were recorded. Concentrations of the phenolic terpenoids used in model were developed on the basis of DOE—the values from CCD have been converted into the analyte concentrations (Table 4).

The exact composition of phenolic terpenoids mixtures used in the experiment is shown in Table S1.

The concentrations of eugenol, carvacrol, and thymol used in the multivariate calibration model were within the linear ranges of univariate calibrations models of these analytes (see Table 3). The voltammograms obtained experimentally for training set are shown in Fig. 4. Two peaks were observed on the curves: first at ~715 mV and second at ~950 mV. The first one increased with the addition of eugenol, while the second one increased with addition of carvacrol or thymol. The curves derived from three independent experiments with the same parameters were averaged and used for further actions.

Table 4 Coded and actual values of the factors (phenolic terpenoid concentration) used in CCD

Type of factor	CCD plan						
	-1.682 (- γ)	-1	-0.5	0.0	+0.5	+1	+1.682 (+ γ)
Factor 1: concentration of eugenol mg/dm ³	0.77	3.39	5.39	7.39	9.39	11.24	13.86
Factor 2: concentration of carvacrol mg/dm ³	0.58	2.54	4.05	5.55	7.05	8.44	10.40
Factor 3: concentration of thymol mg/dm ³	0.73	2.05	3.07	4.10	5.12	6.00	7.32

Fig. 4 Raw data voltammograms registered for the training set, based on CCD: full factorial points (blue), star points (olive), and central points (red); in 0.1 mol/dm³ acetate buffer pH 6.0

The background subtraction procedure (baseline correction) was chosen in such a way that the value of the RMSECV was as small as possible. Different types of baseline subtraction strategies have been tested, including PLS Toolbox function which calculates weighted baseline by the second- or third-degree polynomial with autoscaling procedure and next an innovative ATEB procedure [65]. However, they brought no satisfactory results: RMSECV for raw data was 0.38–0.97 mg/dm³, and after background subtraction 0.58–1.80 mg/dm³, depending on the phenolic terpenoid and the assumed LV number. The reason of the lack of the expected improvement was the fact that simulated background was not in relation to a single peak (as typically in voltammetry), but in relation to two peaks (with one created by carvacrol and thymol). Therefore, in this work, the baseline correction was completely abandoned.

In this work, instead of the background subtracting, various signals pre-processing methods were tested, such as: autoscaling, mean centering, and differentiation by Savitzky–Golay (SG) strategy, with the optimization criterion being the lowest possible value of LV (model complexity), and RMSECV parameters for each of the analyte (predictive correctness). Initially, Savitzky–Golay second derivative with second-degree polynomial (in 21-point frame) with additional autoscaling was chosen as the preliminary pre-processing approach. Figure 5 shows the values

Fig. 5 The values of RMSEC and RMSECV depending on the number of selected LVs, after differentiation by the Savitzky–Golay algorithm (frame of 21 points, polynomial of 2nd deg., 2nd derivative) with autoscaling. The optimal variants are shown in the yellow rectangle

of RMSEC and RMSECV parameters depending on the selected number of LVs and the mentioned pre-processing procedure.

It was observed that selection of one or two latent variables meant the high values of the RMSEC and RMSECV parameters. The choice of 3–6 LVs gave in the effect acceptable level of the RMSEC and RMSECV parameters. The selection of higher number of LV did not improve significantly the model quality. Therefore, the influence of the other signal pre-processing procedures was examined in detail with only 3–6 LVs, resulting in RMSECV values in the range 0.33–1.93 mg/dm³, depending on the phenolic terpenoid and the adopted LV number. It was found that the calibration model obtained after the same signal pre-processing for each analyte was insufficient.

Therefore, the values of the models evaluation parameters for the individual phenolic terpenoids were examined separately: the best five models were selected for each analyte, indicated by the lowest RMSECV value (Table S2). Then, for these selected models, validation with the use of an external test set (validation set) was performed. Finally, the PLS calibration model was built using voltammograms pre-processed by the strategies, as shown in Table 5.

The correctness of model was confirmed by the analysis of graphs presenting the relations of concentration values in relation $\text{predicted} = f(\text{measured})$ (Fig. 6). Relatively low RMSECV in the range of concentrations of each of the analytes (Table 6) also confirmed the correctness of this models.

After confirming correctness of the models, their validation was done using data from the external validation set. Low REP_v error values in the 1.8–5.1% range (Table 6) and good recovery values in the range of: 89.0% (v_2)–101.1% (v_4) for eugenol, 94.5% (v_3)–101.4% (v_8) for carvacrol, and

85.6% (v_1)–108.3% (v_8) (Fig. S4) were obtained. Graphs of concentration values in relation $\text{predicted} = f(\text{measured})$ were shown in Fig. 7a–c. All obtained results allowed to recognize the constructed predictive model as meeting the typical acceptance criteria.

Finally, the validated PLS model was used for the analysis of samples containing only one analyte (Table 7) using data from the external set (Table S1 and Fig. S5).

In each case, the predictive model showed correct content of the analyzed analyte (with satisfactory recovery within

Table 5 Optimal pre-processing used in the PLS model

Type of phenolic terpenoid	Optimal pre-processing used in the PLS model		
	First step—Savitzky–Golay differentiation	Second step	LV number
Eugenol	Second derivative with second-degree polynomial in 21-point frame	Mean centering	3
Carvacrol	First derivative with first-degree polynomial in 15 points frame	Autoscaling	6
Thymol	First derivative with second-degree polynomial in 15 points frame	Autoscaling	5

Fig. 6 Graphs presenting relations of concentration values in relation $\text{predicted} = f(\text{measured})$ in calibration set for: **a** eugenol, **b** carvacrol, and **c** thymol. The light green line is proper ($a=1$, $b=0$, and $r^2=1.0000$) and the black line is fitted (r^2 is on figure)

Table 6 Values of errors: RMSEC, RMSECV, RMSEP, and REP in optimized PLS model; where c —calibration data set, v —validation data set, and h —honey-spiked sample set

Phenolic terpenoid	Concentration range, mg/dm ³	RMSEC, mg/dm ³	RMSECV, mg/dm ³	RMSEP _v , mg/dm ³	RMSEP _h , mg/dm ³	REP _c %	REP _v %	REP _h %
Eugenol	0.77–13.86	0.55	0.71	0.37	0.20	7.4	5.0	2.7
Carvacrol	0.58–10.40	0.20	0.42	0.13	0.83	3.6	1.8	15.0
Thymol	0.73–7.32	0.26	0.38	0.38	0.29	6.3	5.1	7.1

Fig. 7 Plots of concentration values in relation predicted = f (measured) for validation set: **a** eugenol, **b** carvacrol, and **c** thymol; and for honey-spiked sample set: **d** eugenol, **e** carvacrol, and **f** thymol. The light green line is proper and the black line is fitted

Table 7 Qualitative analysis of phenolic terpenoids using the PLS model

Phenolic terpenoid	Added, mg/dm ³			Predicted, mg/dm ³			Recovery ^a %
	Eugenol	Carvacrol	Thymol	Eugenol	Carvacrol	Thymol	
Eugenol	7.39	0.00	0.00	7.31	0.02	0.40	98.92
Carvacrol	0.00	5.55	0.00	0.43	5.64	0.38	101.62
Thymol	0.00	0.00	4.10	0.23	0.09	4.16	101.46

^arecovery if one analyte present in the solution

98.92–101.62%) and the slight presence of other analytes. In this way, it was shown that the presented predictive model allows voltammetric identification of eugenol, carvacrol, and thymol. In particular, distinction of thymol and carvacrol is correct, despite the short distance between peaks (15 mV).

Ultimately, PLS model was used for quantitative analysis of natural objects of complex composition, i.e., the spiked honey samples. Voltammograms of the honey samples with the corresponding amounts of phenolic terpenoids were recorded according to the validation plan (Table S1 and Fig. S6). The values of the obtained

RMSEP_h parameters were in the range 0.20–0.83 mg/dm³ (Table 6). The REP_h coefficients for the honey samples were low in the case of eugenol and thymol determination: 2.7% and 7.1%, respectively; however, for carvacrol—due to the central position of the carvacrol peak, comparing to eugenol and thymol peaks—the REP_h value was higher (15.0%). It should be remembered that the value of REP_h strongly depends on the sample composition—matrix effect. Recovery values for the honey samples were also calculated and were in the range of: 95.7% (h₇)–104.0% (h₁) for eugenol, 80.9% (h₁)–92.5% (h₇) for carvacrol, and

83.0% (h_7)–111.9% (h_1) for thymol (Fig. S4). Graphs of concentration values in relation $\text{predicted} = f(\text{measured})$ are shown in Fig. 7d–f. In this way, the usefulness of the created PLS calibration models in simultaneous determination of phenolic terpenoids in the complex natural samples has been also proved.

Conclusions

It has been proved that there is a possibility of identification and simultaneous determination of eugenol, carvacrol, and thymol using the differential pulse voltammetry (DPV) technique on the BDD electrode in 0.1 mol/dm³ acetate buffer of pH 6.0. The reaction mechanism was tested using the cyclic voltammetry and it was shown that, in each case, it is a diffusion-controlled process in which one electron was involved. It has been demonstrated that the methods of eugenol, carvacrol, and thymol determination were linear in the range of 0.39–14.63, 0.29–11.56, and 0.34–9.52 mg/dm³, respectively, and LOD was 0.02 mg/dm³ for all the considered analytes. The peaks of eugenol, carvacrol, and thymol were not separated from each other, and their location was ~715 mV, ~940 mV, and ~955 mV, respectively. In particular, attention was paid to almost perfect overlapping of the carvacrol and thymol peaks—the difference in their position is only about 15 mV. Therefore, the multivariate strategy was proposed for voltammetric identification and determination of these analytes.

To optimize the PLS model, a low complexity and simple data pre-processing procedures were chosen. The calibration models constructed separately for each analyte provided results of higher quality than in one calibration model for all three phenolic terpenoids. In the concentration ranges of 0.77–13.86, 0.58–10.40, and 0.73–7.32 mg/dm³, the RMSEC values were 0.55, 0.20, and 0.26 mg/dm³, for eugenol, carvacrol, and thymol, respectively. In this way, the correctness of the created model was proved.

The optimized PLS model was used for quantitative analysis of the used phenolic terpenoids. It has been shown that an effective identification of eugenol, carvacrol, and thymol, when only one of these analytes is present in the sample, is possible. The values of recoveries were within the range of 98.92–101.62%. The calibration model was also used for analysis in the complex samples of natural origin which was a solution of honey. REP_h values of 2.7%, 15.0%, and 7.1% were achieved, for eugenol, carvacrol, and thymol, respectively.

The proposed PLS model with relatively low complexity and typical pre-processing procedures can be successfully used for voltammetric identification and simultaneous determination of eugenol, carvacrol, and thymol, although

position of the carvacrol and thymol peaks differs only by 15 mV.

Experimental

Measuring apparatus and software

A multipurpose Electrochemical Analyzer M161 with the electrode stand M164 (both *mtm-anko*, Poland) was used for all the voltammetric measurements. The classical three-electrode quartz cell of 10 cm³ volume was applied. It consisted of the boron-doped diamond electrode (BDDE, $\varphi = 3.0$ mm, Windsor Scientific, UK) as a working electrode, the double-junction reference electrode Ag/AgCl/KCl (3 mol/dm³) with replaceable outer junction (2.5 mol/dm³ KNO₃), and a platinum wire as an auxiliary electrode. The ambient temperature was ca. 23 °C. The *mtm-anko* EAQt software enabled electrochemical measurements, data acquisition, and processing of the results. Sonification of the honey samples was carried out in ultrasonic cleaner IS-2 (manufactured by Intersonic, Poland).

Chemicals, glassware, and samples

Acetic acid and sodium acetate (both pure for analysis, POCh, Poland) were used for the preparation of the supporting electrolyte—0.1 mol/dm³ acetate buffer pH 6.0. The other chemicals were buffers (prepared from pure for analysis reagent, POCh, Poland): acetate (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 6.5), ammonium (pH 10.0), Britton–Robinson (pH 1.75, 3.75, and 11.0), and phosphate (pH 8.0). As the standards, ethanol (99.8%, for HPLC, POCh, Poland) solutions of eugenol (99%, ReagentPlus, Sigma-Aldrich), carvacrol (99%, natural, food grade, Sigma-Aldrich), and thymol ($\geq 98.5\%$, Sigma-Aldrich), were used. For activation of the electrode, 0.5 mol/dm³ solution of sulfuric acid (VI) prepared from 96% H₂SO₄ (pure for analysis, POCh, Poland) was applied. Washing the electrode between the measurements was done using 50% ethanol (obtained from 99.8%, for HPLC, POCh, Poland). For testing of the developed predictive model, the solution of multifloral honey from the local apiary in Galicia was used. 0.20 g of honey was placed in an Eppendorf tube and 800 mm³ of 0.1 mol/dm³ acetate buffer of pH 6.0 was added. The resulting solution was sonicated in ultrasonic cleaner for 5 min (in ambient temperature). All reagents used were prepared using quadruply distilled water (two last stages from quartz). All glassware was first immersed in 6 mol/dm³ nitric acid, and then rinsed repeatedly with distilled water.

Standard procedure of measurements

The BDD electrode was activated by immersing in 0.5 mol/dm³ H₂SO₄ solution and applying the potential of −3.0 V for 15 min once before the whole experiment. The measurements were performed using differential pulse voltammetry (DPV). In the quartz voltammetric cell, 5 cm³ of 0.1 mol/dm³ acetate buffer (pH 6.0) was placed as a supporting electrolyte, and then, adequate volume of the analytes (eugenol, carvacrol, and thymol) according to the experiments plan was added. In the case of honey samples, 25 mm³ of a honey solution was also added in cell. The solution was stirred (ca. 500 rpm) using a magnetic stirring bar. Then, the electrode potential of 2.4 V for 1 s and 0.2 V for 5 s was applied and DP voltammogram was recorded from 0.3 to 1.25 V. The other experimental parameters were as follows: step potential, $E_s = 5$ mV; pulse potential, $dE = 50$ mV; time of potential step and pulse together was 80 ms, i.e., $2 \cdot (t_{\text{waiting}} + t_{\text{sampling}})$, where t_{waiting} and $t_{\text{sampling}} = 20$ ms. The measurement parameters were developed and optimized based on the previous experiments and literature reports [46, 50]. All experiments were carried out in triplicate at ambient temperature about 23 °C. Between the individual measurements, the electrode was washed with 50% ethanol solution.

Software

Processing of the experimental data and calculations were conducted with the home-written procedures in Mathworks Matlab 2014b and Eigenvector Research PLS Toolbox 8.0.

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References

- Kołodziejczyk A (2013) Natural Organic Compounds. PWN, Warsaw
- Wiat C (2014) Lead compounds from medicinal plants for the treatment of neurodegenerative diseases. Academic Press, Netherlands
- Martins MAR, Silva LP, Ferreira O, Schröder B, Coutinho JAP, Pinho SP (2017) J Mol Liq 241:996
- Wang H-F, Yih K-H, Yang C-H, Huang K-F (2017) J Food Drug Anal 25:881
- Lin P-C, Lee JJ, Chang I-J (2016) J Food Drug Anal 24:464
- Tarek N, Hassan HM, AbdelGhani SMM, Radwan IA, Hammouda O, El-Gendy AO (2014) Beni-Suef Univ J Basic Appl Sci 3:149
- Abdelwahab SI, Mariod AA, Taha MME, Zaman FQ, Abdelmaheed AHA, Khamis S, Sivasothy Y, Awang K (2017) Arab J Chem 10:131
- Choi O, Cho SK, Kim J, Park CG, Kim J (2016) Asian Pac J Trop Biomed 6:308
- Starliper CE, Ketola HG, Noyes AD, Schill WB, Chalupnicki MA, Dittman DE (2015) J Adv Res 6:89
- Huang Z, Liu X, Jia S, Luo Y (2017) Food Control 82:316
- Mediouni Ben Jemâa J, Tersim N, Toudert KT, Khouja ML (2012) J Stored Prod Res 48:97
- Sağlam Ö, Dilgin DG, Ertek B, Dilgin Y (2016) Mater Sci Eng, C 60:156
- Kabuto H, Tada M, Kohno M (2007) Biol Pharm Bull 30:423
- Dibazar SP, Fateh S, Daneshmandi S (2014) J Food Drug Anal 22:448
- Zhang S, Zhang M, Fang Z, Liu Y (2017) LWT-Food Sci Technol 75:316
- Cattelan MG, de Oliveira Nishiyama YP, Gonçalves TMV, Coelho AR (2018) Food Microbiol 73:305
- Morshedloo MR, Salami SA, Nazeri V, Maggi F, Craker L (2018) Ind Crops Prod 119:183
- Haberbeck LU, Alberto da Silva Riehl C, de Cássia Martins Salomão B, de Falcão Aragão GM (2012) LWT-Food Sci Technol 46:267
- Xie Y, Wang Z, Huang Q, Zhang D (2017) Ind Crops Prod 108:278
- Kordali S, Cakir A, Ozer H, Cakmakci R, Kesdek M, Mete E (2008) Bioresour Technol 99:8788
- Kokoskova B, Pousova D, Pavela R (2011) J Plant Pathol 93:133
- Daferera DJ, Ziogas BN, Polissiou MG (2003) Crop Prot 22:39
- De Vincenzi M, Stamatii A, De Vincenzi A, Silano M (2004) Fitoterapia 75:801
- Lioliou CC, Gortzi O, Lalas S, Tsaknis J, Chinou I (2009) Food Chem 112:77
- Fadil M, Fikri-Benbrahim K, Rachiq S, Ihssane B, Lebrazi S, Chraïbi M, Haloui T, Farah A (2018) Eur J Pharm Biopharm 126:211
- Komaki A, Hoseini F, Shahidi S, Baharlouei N (2016) J Tradit Complement Med 6:257
- Kačaniová M, Terentjeva M, Vukovic N, Puchalski C, Roychoudhury S, Kunová S, Klůga A, Tokár M, Kluz M, Ivanišová E (2017) Saudi Pharm J 25:1108
- Vaičiulytė V, Ložienė K (2015) Ind Crops Prod 77:491
- Pavel M, Ristic M, Stevic T (2010) J Serbian Chem Soc 75:27
- Lampronti I, Saab A, Gambari R (2006) Int J Oncol 29:989
- Gavanji S, Zaker SR, Nejad ZG, Bakhtari A, Bidabadi ES, Larki B (2015) Integr Med Res 4:112
- Nezhadali A, Nabavi M, Rajabian M, Akbarpour M, Pourali P, Amini F (2014) Beni-Suef Univ J Basic Appl Sci 3:87
- Huang W, Wang J-Q, Song H-Y, Zhang Q, Liu G-F (2017) Asian Pac J Trop Med 10:663
- Kazemi M (2015) J Herb Med 5:217
- Khajeh M, Yamini Y, Sefidkon F, Bahramifar N (2004) Food Chem 86:587
- Sgorbini B, Ruosi MR, Cordero C, Liberto E, Rubiolo P, Bicchi C (2010) J Chromatogr A 1217:2599
- Angelo T, Pires FQ, Gelfuso GM, da Silva JKR, Gratieri T, Cunha-Filho MSS (2016) J Chromatogr B 1022:81
- Safety data sheets for eugenol, carvacrol and thymol (2019) Sigma-Aldrich. <https://www.sigmaaldrich.com>. Accessed 04 Feb 2019
- Cacho JJ, Campillo N, Viñas P, Hernández-Córdoba M (2015) J Chromatogr A 1399:18

40. Ke C, Liu Q, Li L, Chen J, Wang X, Huang K (2016) *J Chromatogr B* 1031:189
41. Tonello N, Moressi MB, Robledo SN, D'Eramo F, Marioli JM (2016) *Talanta* 158:306
42. Yang L, Zhao F, Zeng B (2016) *Electrochim Acta* 210:293
43. Ghiasvand A, Dowlatshah S, Nouraei N, Heidari N, Yazdankhah F (2015) *J Chromatogr A* 1406:87
44. Jiménez-Salcedo M, Tena MT (2017) *J Chromatogr A* 1487:14
45. Kasiotis KM, Tzouganaki ZD, Machera K (2018) *Sci Total Environ* 625:96
46. Stanković DM (2015) *Anal Biochem* 486:1
47. Afzali D, Zarei S, Fathirad F, Mostafavi A (2014) *Mater Sci Eng, C* 43:97
48. Beaudry F, Guénette SA, Vachon P (2006) *Biomed Chromatogr* 20:1216
49. Yun S-M, Lee M-H, Lee K-J, Ku H-O, Son S-W, Joo Y-S (2010) *J AOAC Int* 93:1806
50. Kowalcze M, Jakubowska M (2019) *Anal Chim Acta* 1045:98
51. Michelitsch A, Rittmannsberger A, Hüfner A, Rückert U, Likussar W (2004) *Phytochem Anal* 15:320
52. Elfghi FM (2016) *Chem Eng Res Des* 113:264
53. Cuéllar M, Pfaffen V, Ortiz PI (2016) *J Electroanal Chem* 765:37
54. Silva AC, Lourenço AS, de Araujo MCU (2018) *Food Chem* 266:232
55. Górski Ł, Kowalcze M, Jakubowska M (2019) *J Chemom* 33:e3112
56. Pascual L, Gras M, Vidal-Brotóns D, Alcañiz M, Martínez-Máñez R, Ros-Lis JV (2018) *Sens Actuators, B* 270:231
57. Saurina J, Hernández-Cassou S, Fàbregas E, Alegret S (2000) *Anal Chim Acta* 405:153
58. Abreu REL, Paz JEM, Silva AC, Pontes MJC, Lemos SG (2015) *Fuel* 156:20
59. Sans S, Ferré J, Boqué R, Sabaté J, Casals J, Simó J (2018) *Food Chem* 262:178
60. Zoski CG (2007) *Handbook of Electrochemistry*. Elsevier Science, Netherlands
61. Brett CMA, Brett AMO (1993) *Electrochemistry: principles, methods, and applications*. Oxford Science Publications, Oxford
62. Scholz F (ed) (2010) *Electroanalytical Methods*. Springer, Berlin Heidelberg
63. Biçer E, Arat C (2009) *Croat Chem Acta* 82:583
64. Nicholson RS, Shain I (1964) *Anal Chem* 36:706
65. Górski Ł, Kowalcze M, Jakubowska M (2017) *Electroanalysis* 29:2454

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