

# Study of pH and temperature effect on lipophilicity of catechol-containing antioxidants by reversed phase liquid chromatography

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## ABSTRACT

Lipophilicity of selected antioxidant phytochemicals, including flavonoids, phenolic acids and xanthonoids, was determined by reversed phase high performance liquid chromatography with UV detection (RP-HPLC-UV). The analyses run at different temperature and pH conditions in isocratic mode suggested that lipophilicity as distribution coefficient ( $\log D$ ) between aqueous and organic phase decreases with increasing temperature. For all studied compounds,  $\log D$  changes slightly at lower pH and much decreases at a given pH range corresponding to the strongest dissociation by which dissociation constant ( $pK_a$ ) was estimated experimentally as well as their bioavailability in gastrointestinal pH conditions were predicted. Thermodynamic parameters ( $\Delta_r H^\circ$ ,  $\Delta_r S^\circ$ ,  $\Delta_r G^\circ$ ) demonstrated the increase in energy of transfer from the aqueous to the organic phase at higher pH thus decrease in lipophilicity. Experimental models of pH and temperature effect obtained by multiple regressions underlined their significant impact ( $p < 0.05$ ) on lipophilicity. The provided lipophilicity profiles and  $pK_a$  data are useful descriptors to assess the affinity of the studied compounds for biological membranes, enzymes, carriers and target sites. Furthermore, the obtained result may be useful in the design of functional and medical foods, especially in order to increase the bioavailability of bioactive constituents, such as phenolic compounds, based on their lipophilic and acid-base character.

### Keywords:

RP-HPLC

Lipophilicity

Dissociation constant

*o*-Diphenols

Antioxidants

Temperature effect

## 1. Introduction

Antioxidant (AOX) phytochemicals are important bioactive compounds mainly provided with plant-based foods, especially with functional foods and diet supplements, for various non-nutritional purposes including prevention of oxidative stress and protection against chronic diseases [1]. Oxidative stress is defined as excessive production of reactive oxygen or nitrogen species (ROS/RNS) that cannot be counteracted by AOXs, thus redox imbalance between generation and depletion of ROS/RNS [2]. These species are highly reactive atoms, ions or molecules including free radicals and non-radical species such as superoxide, singlet oxygen ( $^1O_2$ ), hydroxyl radical ( $HO^\bullet$ ), hydrogen peroxide ( $H_2O_2$ ), alkoxy radical ( $RO^\bullet$ ), peroxy radical ( $ROO^\bullet$ ), peroxyxynitrite ( $ONOO^-$ ), nitrogen dioxide ( $NO_2$ ) and dinitrogen trioxide ( $N_2O_3$ ) [2,3]. They are generated as reaction intermediates during aerobic process of energy production in the cell as well as in protective mechanism against pathogens and exposure to pollutant such as toxins, tobacco smoke, UV-radiation and ozone [3,4]. The produced free radicals can

attack neighboring lipids, proteins and nucleic acids especially during imbalance between their production and scavenging [3,5].

The provision of optimal concentration of AOX plays a key role in the stabilization and inactivation of free radicals before they attack cell thus decrease substrate oxidation that reduces the risk of DNA mutation and malignant transformation [2,6]. The existing endogenous AOXs like glutathione are supported by various exogenous antioxidant phytochemicals from food diet that are easily absorbed by cells to boost the mechanism of free radical scavenging [6,7].

A large number of phytochemicals has been up to date characterized by *in vitro* and *in vivo* antioxidant activity measurements of both pure compounds and their complex mixtures in the raw material i.e. plant extracts [8–10]. However, there is still limited number of studies on factors affecting radical scavenging capacity of antioxidant that mainly include (i) the reactivity of AOX towards free radical, (ii) stoichiometry factor, (iii) possibility of secondary reactions in which product of radical scavenging can be involved, and (iv) lipophilicity as a fact of absorption of AOXs in cellular compartments where free radicals

are generated [11]. Although several papers have been focused on the lipophilicity and solubility of phenolic antioxidants [12–14], data on the dependence of both properties on temperature and pH are still scarce and unavailable.

Phenolic compounds scavenge free radicals by hydrogen donation leading to the formation of phenoxyl radical that need to be stabilized either by dimerization or electron resonance and then excreted [15]. Otherwise, the generated phenoxyl radicals with high lipophilicity may be toxic and cause lipid cell peroxidation [16–18]. It was found that optimal absorption occurs for compounds with lipophilicity in the range from 1 to 3 defined as partition coefficient ( $\log P$ ) and becomes less significant at a value higher than five [19]. The amount of ionic species, which is correlated with dissociation constant and inversely proportional to lipophilicity, plays a key role in absorption, distribution, metabolism, elimination (ADME) processes and the bioavailability of phenolic compounds. Therefore, temperature and pH conditions may affect lipophilicity of these compounds [20,21]. Catechol-containing polyphenols are one of the phenolic compounds ortho-substituted with a strong electron donor group ( $-\text{OH}$ ) thus have higher reactivity in hydrogen atom transfer (HAT) processes [22]. *O*-diphenols react with free radicals to produce nontoxic quinones (as shown in Fig. 1) that reduces the risk of secondary oxidation reactions. Therefore, *o*-diphenols that are characterized by moderate lipophilic properties can be antioxidants of potential interest for nutritional and medical applications [23]. In this context, lipophilicity profile and acid-base character of phenolic phytochemicals are important parameters in the design of functional and medical foods to ensure they reach the target site of action in the body [24].

Taking the above into account, the aim of this work is to determine lipophilicity of different catechol-containing polyphenols from 5 subgroups (flavan-3-ols, flavonols, flavones, phenolic acids, xanthonoid) and to evaluate effect of pH and temperature on their lipophilicity. In many research works, lipophilicity is expressed as octanol-water partition coefficient determined using direct methods, especially shake flask method [25,26]. However, indirect chromatographic approaches are emerging methods used to describe lipophilicity as partition or distribution of a compound between aqueous mobile phase and organic stationary phase [14,27]. In this study lipophilicity was determined as distribution coefficient between a aqueous and hydrophobic phases ( $\log D$ ) using RP-HPLC [28]. Temperature effect and experimental thermodynamic evaluation of compound transfer from aqueous to organic phase was carried out by lipophilicity determination at different temperatures. In addition, effect of pH with estimation of the strongest dissociation constants ( $\text{pK}_a$ ) and bioavailability of polyphenols under physiological conditions were evaluated.

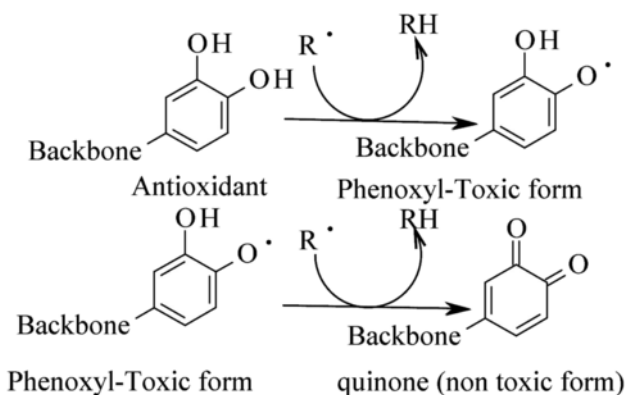


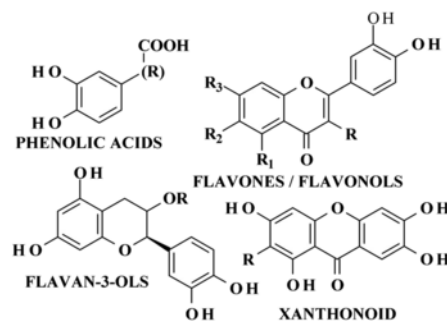
Fig. 1. Illustration of reaction of *o*-diphenolic antioxidant on free radical leading to formation of non-toxic quinone.

## 2. Materials and methods

### 2.1. Chemicals

All the organic solvents and buffering reagents including methanol, dimethyl sulfoxide (DMSO), formic acid (98–100%), glacial acetic acid, ammonia solution (25%), and ammonium acetate were of HPLC grade and purchased from Sigma Aldrich (Poznań, Poland). Uracil was used as an unretained compound to determine column dead time ( $t_0$ ). Ultrapure water used throughout this study was purified with a Milli-Q Gradient A10 System (Millipore, Bedford, MA, USA) with a resistivity of  $18.2 \text{ M}\Omega \text{ cm}$  at  $25^\circ \text{C}$ . Buffer solutions pH4.01, pH7.01 and pH10.01 were used for pH meter calibration.

The studied *o*-diphenolic compounds were randomly selected among phenolic acids, flavonoids, and xanthonoids. All of them were of analytical or HPLC grade with purity higher than 99%. They were purchased from Sigma-Aldrich and Extrasynthese (Genay, France). General structures and typical substitution for each of the compound are shown in Fig. 2. Aliquots of each test compound were weighed and dissolved in DMSO to obtain standard stock solutions with the concentration of  $2 \text{ mg mL}^{-1}$ . For mobile phases, water and methanol both with the addition of buffer to obtain suitable pH have been applied [29]. A buffer of pH2.8 was prepared by addition of formic acid to have 0.1% (v/v) in each component of mobile phase. Buffer solutions of pH7.4 and higher were made by dissolving 0.05 mol of ammonium acetate in 1 L of phase adjusted to a given pH using ammonia solution, while buffers with a pH below 7.4 were prepared by acidifying 50 mM ammonium acetate of mobile phase using acetic acid.



Subgroup	R	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	Compound
Phenolic acids	R <sup>1</sup>	-	-	-	Chlorogenic acid (CA)
	-	-	-	-	Protocatechuic acid (PA)
Flavan-3-ols	H	-	-	-	(-) Epicatechin (EC)
	Gallyl	-	-	-	Epicatechin gallate (ECG)
Flavones	H	OH	Gl	OH	Isoorientin (ISR)
	H	OH	H	OH	Luteolin (LUT)
Flavonols	OH	OH	H	OH	Quercetin (QRC)
	O-Rut <sup>2</sup>	OH	H	OH	Rutin (RUT)
Xanthonoid	Gl <sup>3</sup>	-	-	-	Mangiferin (MNG)

<sup>1</sup> [(prop-2-enoyl)oxy]-1,4,5-trihydroxycyclohexane

<sup>2</sup> Rutinose

<sup>3</sup> Glucose

Fig. 2. Structures of studied catechol-containing phenolic compounds.

## 2.2. Instrumentation and HPLC conditions

Chromatographic analyzes were performed using an Agilent 1100 series HPLC system equipped with degasser (G1379B), binary pump (G1312A), autosampler (G1313A), thermostat (G1316A) and diode array detector (G1315A). System control, data acquisition and processing were carried out with Agilent Chemstation software. For  $\log D$  determination a Purospher STAR RP-18 endcapped column (125 mmL  $\times$  3 mm i.d.; 5  $\mu$ m particle size) was used.

Lipophilicity was determined using OECD 117 method, [30] with modification based on Snyder-Soczewinski linear solvent strength (LSS) model [31]. Test solutions were prepared in chromatographic vials by two-fold dilution of stock solutions to get 1 mg mL<sup>-1</sup>. Pre-runs were performed for each compound in order to select optimal absorption wavelengths. Absorbance spectra were recorded between 190 and 400 nm every 2 s with a bandwidth of 4 nm, while the chromatograms were monitored at 280, 340, 360, and 254 nm for flavan-3-ols, flavones, flavonols, and xanthonoid, respectively. The wavelengths used to record chromatograms of phenolic acids were 254 nm for PA and 325 nm for CA. The retention times ( $t_R$ ) of each compound were measured under isocratic mode using a mixture of methanol and buffer in different proportions. The variation of methanol was 5% increment except for HPLC experiments conducted with percentage of methanol ( $\varphi$ ) below 15% where the increment of 2.5% was used. Analyzes were performed at different temperatures (20 °C, 25 °C, 30 °C and 37 °C) and pH conditions (2.8, 4.0, 5.6, 7.4, 8.6, and 10.2). For accurate pKa calculations, additional chromatographic runs under pH 5.0 and 5.4 for phenolic acids and pH 9.6 for flavan-3-ol were carried out at 25 °C. All measurements were carried out in duplicate using flow rate of 0.7 mL min<sup>-1</sup> and the injection volume of 2  $\mu$ L. The results obtained for five different methanol concentrations were used to obtain LSS equation which intercept is the main lipophilicity descriptor corresponding to 0% organic modifier and denoted as  $\log k_w$ .

## 2.3. Data analysis and calculation procedures

For each pH of mobile phase and different temperatures, the values of logarithm of retention factor ( $\log k$ ) were calculated and extrapolated to 0% organic modifier (pure water as the mobile phase) in LSS Eq. (2) to obtain  $\log k_w$  [32].

$$\log k = \log k_w - S \quad (1)$$

The phase ratio of HPLC column ( $\emptyset$ ) known as the ratio of the volume of stationary phase ( $V_s$ ) to the void volume ( $V_m$ ) was determined using Eq. (2) as suggested by other authors [33].

$$\log \emptyset = \frac{\log k_j \log K_{owj} - \log k_i \log K_{owi}}{\log K_{owj} - \log K_{owi}} \quad (2)$$

where  $\log k_i$ ,  $\log k_j$  are experimentally determined retention factors of two hydrocarbons from HPLC column certificate, and  $\log K_{owi}$ ,  $\log K_{owj}$  are their partition coefficients. The obtained  $\emptyset$  value was used to calculate  $\log D$  from  $\log k_w$  based on Eq. (3).

$$\log k_w = \log D + \log \emptyset \quad (3)$$

Partition coefficient ( $K_{ow}$ ) defined as the ratio of the concentration of a compound in unionized form ( $C_{i\text{-neutral}}$ ) in the organic phase to its concentration in the aqueous phase ( $C_{i\text{-neutral}}$ ) is commonly calculated by formula (4). However, this relationship is significant only at a lower pH for ionizable compounds, and thus the distribution constant ( $D$ ) of Eq. (5) is preferred to express the partition of species between water and other immiscible phase [21].

$$K_{ow} = \frac{\sum_i^n C_{i\text{-neutral}}}{\sum_i^n C_{i\text{-neutral}}} \quad (4)$$

$$D = \frac{\sum_i^n C_{i\text{-neutral}} + \sum_i^n C_{i\text{-ionized}}}{\sum_i^n C_{i\text{-neutral}} + \sum_i^n C_{i\text{-ionized}}} \quad (5)$$

It is assumed that there is no significant ionization in organic phase thus  $D$  can be expressed by a simplified Eq. (6) [21]. The value of  $\log D$  is equal to  $\log K_{ow}$  when the concentration of ionized forms is zero. It decreases with increasing concentration of ionized forms in accordance with Eq. (7). In other words, the inverse fraction of unionized forms increases.

$$D = \frac{\sum_i^n C_{i\text{-neutral}}}{\sum_i^n C_{i\text{-neutral}} + \sum_i^n C_{i\text{-ionized}}} = K_{ow} * \left( 1 + \frac{\sum_i^n C_{i\text{-ionized}}}{\sum_i^n C_{i\text{-neutral}}} \right)^{-1} \quad (6)$$

$$\log D = \log K_{ow} - \log \left( \frac{\sum_i^n C_{i\text{-ionized}} + \sum_i^n C_{i\text{-neutral}}}{\sum_i^n C_{i\text{-neutral}}} \right) \quad (7)$$

### 2.3.1. Procedures for pH effect calculations

According to the theory of Brønsted-Lowry, the acid-base character was considered with concept of a compound  $H_nA$  dissociating stepwise by transferring  $nH^+$ , from step  $i = 1$  to step  $n$  with various acidity constants (pKa) in aqueous phase [21,34]. However great effect is observed with strong dissociation corresponding to maximal change of  $\log D$  as a function of pH.

Therefore, only one pKa was considered significant that involves the strongest dissociation constant  $K_a$  in Eq. (8). At each pH condition, a fraction of protonated form is given by Eq. (9) and the change of  $\log D$  with pH is found by replacing Eq. (9) in Eq. (7) to obtain Eq. (10).

$$K_a = [H^+] [A^-] / [HA] \quad (8)$$

$$f_u = 1 / (1 + 10^{pH-pKa}) \quad (9)$$

$$\log D = \log K_{ow} - \log [1 + 10^{(pH-pKa)}] \quad (10)$$

Since phenolic compounds are weak acids, retention factor ( $k_w$ ) calculated based on LSS at experimental pH below 3 was corresponded to retention factor of neutral form ( $k_{HA}$ ). The one determined at pH higher with 1 (2) unit(s) than pKa was considered as retention factor of ionized form ( $k_{A^-}$ ). The relationship between the retention factor and pH is expressed by Eq. (11) and subsequently pKa was calculated using Eq. (12) based on the experimental data [35–37]. Under physiological conditions, the percentage ( $\alpha_i$ ) of available protonated and deprotonated forms were estimated using the Eq. (13).

$$k = \frac{k_{HA} + (10^{pH-pKa}) k_{A^-}}{1 + 10^{pH-pKa}} \quad (11)$$

$$pKa = pH - \log \left( \frac{k_{HA} - k}{k - k_{A^-}} \right) \quad (12)$$

$$\alpha_i = \frac{[H^+]^{n-i} K_{ai}}{[H^+]^{n-0} K_{a0} + [H^+]^{n-1} K_a} \quad (13)$$

where for protonated form ( $n = 0$  and  $K_{ai} = 1$ ) while for deprotonated species ( $n = 1$  and  $K_{ai} = 10^{-pK_{ai}}$ ) [38].

### 2.3.2. Procedures for temperature effect calculation

Gibbs free energy ( $\Delta_r G^\circ$ ) was used to describe the energy of transfer of a compound from water to organic phase with equilibrium constant  $K$  at given temperature.

Thermodynamic parameters that are related to energy of transfer to organic phase, including entropy of transfer ( $\Delta_r S^\circ$ ) and enthalpy of transfer ( $\Delta_r H^\circ$ ), were calculated based on van't Hoff Eq. (14).

$$\log K = \left( -\frac{\Delta H^\circ}{2.303R} \right) \frac{1}{T} + \frac{\Delta S^\circ}{2.303R} \quad (14)$$

Therefore, experimental results of  $\log D$  at different temperatures were plotted versus inverse temperature. The slope ( $-\Delta H^\circ/2.303R$ ) and intercept ( $\Delta S^\circ/2.303R$ ) of this correlation  $\log D = f(1/T)$  allowed to determine  $\Delta_r H^\circ$  and  $\Delta_r S^\circ$ , respectively [13,39].

### 2.3.3. Uncertainty and significance of results

The uncertainty for  $\log k_w$  and  $\Delta_r S^\circ$  were estimated in a value of ( $y$ ) corresponding to intercept ( $x = 0$ ) using model for uncertainty of linear least square regression, as shown in Eq. (15). Uncertainty of  $\Delta_r H^\circ$  was estimated from standard uncertainty of the slope of  $\log D = f(1/T)$ . Uncertainty of pKa was estimated by propagation relation (16) including components of pKa Eq. (12). The contribution of pH meter calibration, and runs precision were negligible [40].

$$u_y^2 = S_{y,x_p}^2 + S_{y,x_p}^2 \left( \frac{1}{n} + \frac{(x_p - \bar{x})^2}{SS_{xx}} \right) \quad (15)$$

$$\left( \frac{u_{\bar{x}}}{\bar{x}} \right)^2 = \sum_i^n \left( \frac{u_i}{\bar{x}_i} \right)^2 \quad (16)$$

where  $y$  stands for  $\log k_w$  and  $\log D$ , while  $x_p$  stands for  $\varphi = 0$  and  $1/T = 0$  for Eqs. (1) and (14), respectively. The values of standard deviation  $SS_{xx}$  and  $S_{y,x}$  were calculated using Excel functions  $DEVSQ(x_1:x_n)$  and  $STEYX(y_1:y_n, x_1:x_n)$ , respectively. The obtained results were compared with literature values at significance levels of  $\alpha = 0.318$ ,  $\alpha = 0.046$ , and  $\alpha = 0.010$  corresponding to normal probability distribution of 68.2%, 95.4% and 99% confidence intervals, respectively [40,41].

### 2.3.4. Evaluation of combined effect of pH and temperature

Effect of temperature and pH on lipophilicity was evaluated using three level factorial design involving nine experimental responses for each compound. Matrices were created as shown in Table 1 for each compound and experimental results of  $\log D$ . Data were analyzed using

**Table 1**  
Three level factorial design matrices for pH and temperature effect evaluation.

Levels	T [°C]			pH		
	-1	0	+1	-1	0	+1
CA	20	25	30	2.8	4.0	5.7
PA	20	25	30	2.8	4.0	5.0
MNG	20	25	30	2.8	5.7	8.6
EC	20	25	30	4.0	7.4	10.2
ECG	20	25	30	4.0	7.4	10.2
QRC	20	25	30	2.8	5.7	8.6
RUT	20	25	30	2.8	5.7	8.6
LUT	20	25	30	2.8	5.7	8.6
ISR	20	25	30	2.8	5.7	8.6

NCSS 12 statistical software. The temperature was expressed in Celsius degrees for 3D surface response while it was changed to absolute temperature in multiple regression modeling. Quadratic regression model was used to express response ( $\log D$ ) as a function of the independent variables (pH, T) for each compound at significance level of  $\alpha = 0.05$  according to Eq. (17).

$$Y = b_0 + \sum_{i=1}^k b_i x_i + \sum_{i=1}^k b_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^k b_{ij} x_i x_j + e \quad (17)$$

where  $Y$  is determined response ( $\log D$  value);  $x_i$  and  $x_j$  are pH and temperature variables;  $k$  is a number of variables ( $k = 2$ );  $e$  is a random error;  $b_0$ ,  $b_i$ ,  $b_{ii}$ ,  $b_{ij}$  are the regression coefficients of variables for intercept, linear, quadratic and interaction terms, respectively [42,43].

## 3. Results and discussion

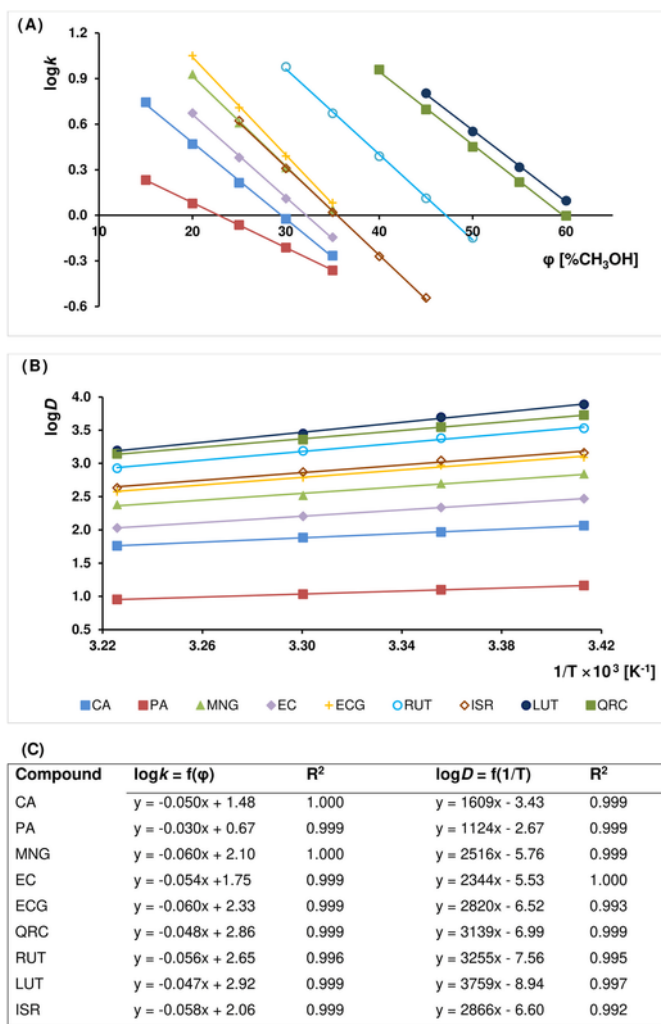
For each pH and temperature,  $\log k_w$  was found as intercept of  $\log k = f(\varphi)$  based on LSS Eq. (1). The results of retention factor as a function of methanol percentage in mobile phase obtained at pH 2.8 and 37 °C are shown in Fig. 3A and C. All of the experimental data for LSS equations obtained under various pH and temperature conditions were summarized in Supplementary material (Table S1). Lipophilicity determination by RP-HPLC-DAD was precise ( $RSD < 0.01$ ) and uncertainty of  $\log k_w$  was  $< 10\%$  at significance level of  $\alpha = 0.32$ .

Lipophilicity as distribution coefficient  $\log D$  was obtained using Eq. (3) where the phase ratio was calculated according to Eq. (2). The retention factors of two compounds indicated on the HPLC column certificate ( $k_i = 1.8$  for toluene and  $k_j = 2.4$  for naphthalene) and their partition coefficients from online database of Pubchem ( $\log K_{ow,i} = 2.73$  and  $\log K_{ow,j} = 3.35$ ) were used for  $\varnothing$  calculation. The obtained value of 0.53 for  $\varnothing$  suggests to be in the range (0.079–0.665) reported for RP-HPLC columns and is close to upper limit that is typical for C18 columns [33].

The results of  $\log D$  obtained at different temperatures for pH 2.8 and 7.4 are presented with 68.2% confidence interval (mean  $\pm 1\sigma$ ) in Table 2. It was observed that at the same pH, lipophilicity decreases with increasing temperature while at the same temperature it was found to be lower at high pH. The change in structure for the same subgroup of phenolic compounds correlates with change in lipophilicity. For flavan-3-ols, esterification of —OH group of epicatechin by gallic acid introduces an additional phenyl group that increases lipophilicity of epicatechin gallate. In case of phenolic acids, the increase in carbon chain makes chlorogenic acid more lipophilic than protocatechuic acid [44]. For flavones and flavonols, the additional —OH group on quercetin makes it less lipophilic than luteolin, and lipophilicity decreases more for isoorientin that has glucose moiety. Although rutin has many additional —OH groups from sugar moieties (glucose and rhamnose), its lipophilicity is higher than isoorientin due to increased number of carbon in structure, especially the methyl group (—CH<sub>3</sub>) on rhamnose. Based on the results, it can be stated that the aglycones are more lipophilic than any conjugate, especially glycoside. In addition, lipophilicity results were similar to those reported by other authors [13,19,45] using different methods, including electrokinetic chromatography with dodecyl (C12) stationary phase [46].

The correlation between  $\log D$  and pH was evaluated and the trendlines obtained at 25 °C are shown in Fig. 4. The pKa values were calculated using Eq. (15) and the results together with significantly similar data found in literature are presented in Table 3.

Previously, it was reported that acidity constant of simple phenols ranged from 8 to 10 [47]. Moreover, pKa changes are monitored by electron donor's substituents causing decrease in acidity including alkyls and —OH and electron withdrawers including —COOH group that increases acidity. It was found that at standard temperature acidity



**Fig. 3.** Lipophilicity descriptors as a function of various parameters: (A) linear correlation of RP-HPLC retention behavior ( $\log k$ ) with increasing percentage organic modifier ( $\phi$ ), (B) distribution coefficient ( $\log D$ ) plotted vs. inverse temperature ( $1/T$ ), and (C) the equations describing both relationships  $\log k = f(\phi)$  and  $\log D = f(1/T)$ . This figure shows exemplary results obtained at pH 2.8.

decreases in the order of phenolic acids > xanthonoid > flavones and flavonols > flavan-3-ols thus pKa values in opposite order. In fact this suggests that presence of carboxylic acid group near phenolic ring decreases pKa for protocatechuic acid, while it decreases even more for chlorogenic acid by additional electron withdrawer carbonyl group from prop-2-enoyl. The xanthone backbone of mangiferin has electron withdrawing group deriving from salicylate that is suggested to affect

**Table 2**

LogD results for o-diphenols at various temperatures at acidic and physiological pH.

T [°C]	logD at pH 2.8				logD at pH 7.4				Similar results ( $p > 0.01$ )
	20	25	30	37	20	25	30	37	
CA	2.06 ± 0.02	1.97 ± 0.02	1.88 ± 0.02	1.76 ± 0.03	1.18 ± 0.01	1.08 ± 0.02	1.03 ± 0.02	0.91 ± 0.01	–
PA	1.16 ± 0.02	1.10 ± 0.01	1.03 ± 0.01	0.95 ± 0.01	–	–	–	–	0.76; 0.80 [13]
MNG	2.84 ± 0.04	2.70 ± 0.04	2.52 ± 0.04	2.33 ± 0.05	2.07 ± 0.04	1.94 ± 0.10	1.80 ± 0.04	1.62 ± 0.10	2.73 [19]
EC	2.47 ± 0.03	2.34 ± 0.03	2.21 ± 0.04	2.03 ± 0.04	2.49 ± 0.20	2.35 ± 0.03	2.22 ± 0.03	2.05 ± 0.04	–
ECG	3.09 ± 0.03	2.97 ± 0.03	2.79 ± 0.03	2.57 ± 0.04	3.10 ± 0.03	2.99 ± 0.03	2.81 ± 0.04	2.61 ± 0.04	–
QRC	3.73 ± 0.06	3.55 ± 0.06	3.36 ± 0.12	3.14 ± 0.05	3.59 ± 0.04	3.35 ± 0.05	3.20 ± 0.16	2.88 ± 0.03	–
RUT	3.53 ± 0.07	3.38 ± 0.14	3.19 ± 0.09	2.93 ± 0.05	3.44 ± 0.05	3.26 ± 0.10	3.07 ± 0.05	2.76 ± 0.05	2.59 [46]
LUT	3.89 ± 0.07	3.70 ± 0.07	3.45 ± 0.11	3.19 ± 0.06	3.75 ± 0.06	3.44 ± 0.06	3.24 ± 0.11	2.97 ± 0.06	3.00 [46]
ISR	3.16 ± 0.06	3.04 ± 0.11	2.87 ± 0.07	2.63 ± 0.04	3.08 ± 0.05	2.88 ± 0.10	2.73 ± 0.05	2.55 ± 0.05	–

its pKa making it lower than phenol ( $pK_a < 10$ ) [48]. Flavones and flavonols including rutin, luteolin, quercetin, isoorientin, have also ketone carbonyl group on benzopyran-4-one that plays an electron-withdrawing role thus increase acidity to more acidic than phenol. Flavan-3-ols, epicatechin and epicatechin gallate, having benzopyran-4-ol group exhibits higher pKa than flavones due to additional electron donor group ( $-\text{OH}$ ) on benzopyran that decreases for epicatechin gallate due to esterified  $-\text{OH}$  group. The impact of structure on acidity of analyzed compounds is evident and their pKa increases in the order of CA, PA < MNG < LUT, RUT, QRC, ISR < ECG, EC.

In fact, ionization of a bioactive compounds decrease their lipophilicity thus their bioavailability is reduced due to the lower membrane permeability for charged species of a compound. The pH range from 1 to 8 was reported for digestive tube of humans with particular range of 5.5 to 7.5 for intestine [20,21]. The availability of species were calculated using pKa values determined at 37 °C in Eq. (13). Fig. 5 illustrates the predicted availability of protonated and deprotonated forms at physiological temperature expressed as a percentage. With exception of phenolic acids, the bioavailability of studied compounds is higher than 73.3% at pH 5.5, while at pH 7.5 it decreases below 60.2% and even reaches 2.7% for some of the compounds (e.g. ISR). Under intestinal conditions phenolic acids are mainly in their deprotonated form (< 2% in uncharged form) that have negative effect on passive permeability across the membrane. Among studied antioxidants, ECG is expected to be the most absorbable followed by QRC and RUT (Fig. 5).

Temperature effect on lipophilicity was evaluated based on  $\log D = f(1/T)$  plots as shown in Fig. 3B and C. It was observed that lipophilicity decreases with increasing absolute temperature. Thermodynamic function enthalpies, entropies and Gibbs energies of transfer between water and organic stationary phase at pH 2.8 and pH 7.4 for each compound are presented in Table 4. Similarly, as reported by Noubigh et al. [13], the thermodynamic functions of transfer of phenolic compounds were negative. The results suggest a spontaneous ( $\Delta_r G^\circ < 0$ ) and ordered inter-phase transfer from the aqueous to the organic phase ( $\Delta_r H^\circ < 0$ ,  $\Delta_r S^\circ < 0$ ). Therefore, the passive transport through lipid membranes for the studied compounds is highly possible. Although the transport is spontaneous at lower pH, the increase of charged forms at higher pH has a negative effect and may cause some compounds to not pass through the lipid bilayers (e.g. PA).

In order to present the relationship between independent variables (pH and temperature conditions) and dependent ones ( $\log D$  of antioxidant), the regression models were generated and illustrated as 3D response surface plots in Fig. 6 and Table 5, respectively. The models obtained according to Eq. (17) with 95% confidence level adequately represent the experimental data with the coefficient of determination ( $R^2$ ) of at least 0.9925. They indicate a significant effect of pH and absolute temperature on  $\log D$  for all compounds ( $p < 0.05$ ). In fact, higher lipophilicity is observed at lower pH and temperature. There is a sud-

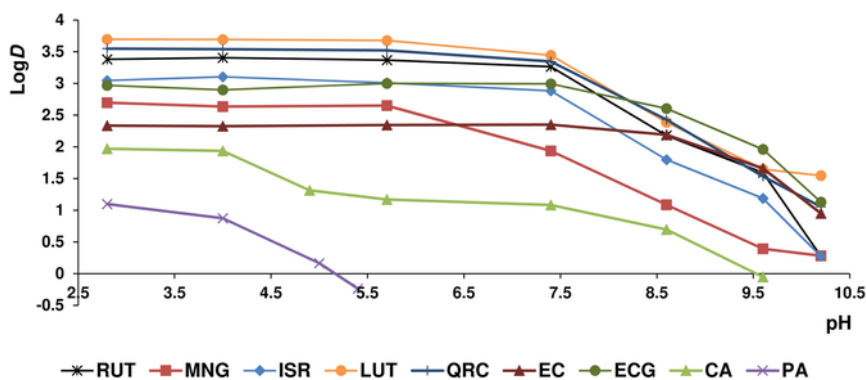


Fig. 4. Effect of pH on lipophilicity at standard temperature (25°C) presented as: (A) variations of distribution coefficient, and (B) the highest logD change in the investigated pH range.

Table 3  
pKa values (mean  $\pm$  1 $\sigma$ ) obtained for *o*-diphenols at different temperatures.

	pKa				pKa at 25°C from literature		
	20°C	25°C	30°C	37°C	$p > 0.32$	$p > 0.05$	$p > 0.01$
CA	4.49 $\pm$ 0.15	3.97 $\pm$ 0.13	3.46 $\pm$ 0.11	2.92 $\pm$ 0.28	3.91 $\pm$ 0.50 [46]	–	–
PA	4.55 $\pm$ 0.12	4.17 $\pm$ 0.16	3.89 $\pm$ 0.10	3.79 $\pm$ 0.20	4.16 [49]	4.35[49]	4.45 $\pm$ 0.10 [46]
MNG	6.70 $\pm$ 0.45	6.66 $\pm$ 0.31	6.64 $\pm$ 0.52	6.60 $\pm$ 0.44	6.52 $\pm$ 0.06 [46]	–	–
EC	8.95 $\pm$ 0.20	8.93 $\pm$ 0.48	8.48 $\pm$ 0.18	7.01 $\pm$ 0.24	8.91 $\pm$ 0.23 [50]	–	9.54 $\pm$ 0.10 [46]
ECG	8.53 $\pm$ 0.17	8.47 $\pm$ 0.20	8.46 $\pm$ 0.20	7.68 $\pm$ 0.21	–	–	–
QRC	7.51 $\pm$ 0.17	7.50 $\pm$ 0.28	7.46 $\pm$ 0.46	7.37 $\pm$ 0.16	7.30 [51]	7.10 $\pm$ 0.12 [50]	6.31 $\pm$ 0.40 [46]
RUT	7.40 $\pm$ 0.23	7.36 $\pm$ 0.43	7.35 $\pm$ 0.28	7.30 $\pm$ 0.23	7.35 $\pm$ 0.02 [52]	7.1 [51]; 6.84 $\pm$ 0.60 [52]	6.17 $\pm$ 0.40 [46]
LUT	7.82 $\pm$ 0.19	7.32 $\pm$ 0.23	7.26 $\pm$ 0.38	7.15 $\pm$ 0.23	–	–	6.50 $\pm$ 0.40 [46]
ISR	7.30 $\pm$ 0.19	7.26 $\pm$ 0.40	6.91 $\pm$ 0.33	5.94 $\pm$ 0.25	–	–	–

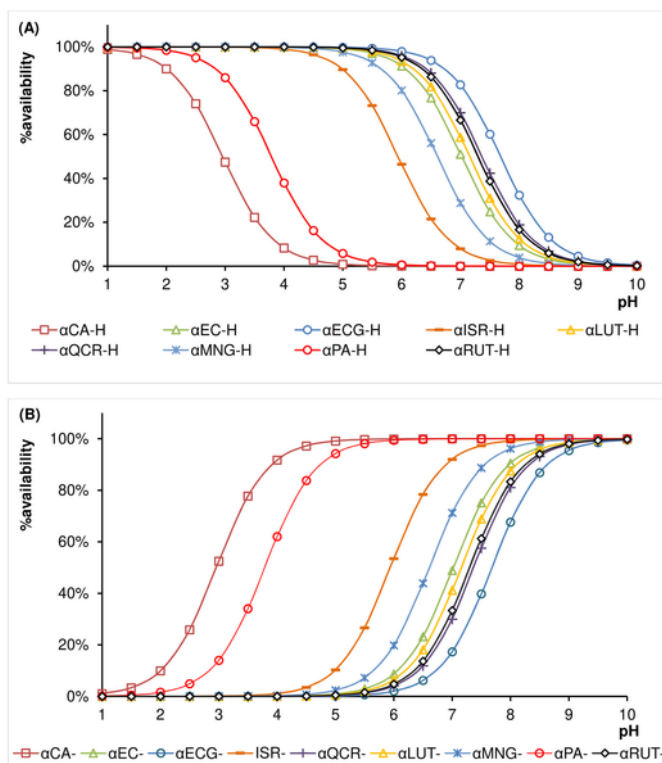


Fig. 5. Predicted availability of protonated (A) and deprotonated (B) forms of *o*-diphenols in gastrointestinal pH conditions at 37°C.

den decrease in lipophilicity at a pH close to pKa value in the pH range of  $pK_a - 1 < pH < pK_a + 1$ , which corresponds to the curvature surface area visible on the 3D graph in Fig. 6. LogD becomes lower at higher

pH and temperature. The effect is much more significant for phenolic acids, where PA was found to be fully hydrophilic at  $pH > 5.5$ , while lipophilicity of CA becomes quite lower under the same conditions. The effects are observed in the following order: phenolic acids > xanthonoid > flavones and flavonols > flavan-3-ols.

#### 4. Conclusion

Our findings indicate that lipophilicity of studied catechol-containing antioxidants is in optimal logD range of 1–3 at physiological temperature and pH conditions with exception of procatechuic acid, which is hydrophilic at high pH. Therefore, their easy transport, absorption and removal in aqueous and fatty organs are highly possible and make them potential natural bioactive compounds of therapeutic interest. Compound structure has a significant effect on lipophilicity. The introduction of sugar moiety, OH and COOH into main backbone decreases logD, which underlines relatively low lipophilicity of phenolic acids, while the additional alkyl or aryl group increases logD. Lipophilicity of compounds with high pKa values does not change significantly at lower pH. The highest changes in lipophilicity were observed in the pH range close to the strongest dissociation constant ( $pK_a - 1 < pH < pK_a + 1$ ). On the other hand, logD was inversely proportional to the increase in temperature. Furthermore, obtained regression models show significant effect of both pH and temperature ( $p < 0.05$ ). The results of RP-HPLC-DAD determination are repeatable and in good agreement with other data available in literature. To the best of our knowledge, some of the studied antioxidants were characterized for the first time in terms of lipophilic and acid-base character, especially under various pH and temperature conditions. Newly provided values of distribution coefficient and pKa can be useful descriptors to assess the affinity of the molecule for low dielectric media (e.g. biological membranes, enzymes, carriers and target sites). The obtained result may be useful in the design of functional and medical foods, especially in order

**Table 4**

Values of the standard thermodynamic parameters of transfer of *o*-diphenols from the aqueous phase to the organic phase, at acidic and physiological pH. Molar Gibbs energy of transfer ( $\Delta_r G$ ) at physiological temperature was also presented.

	pH 2.8			pH 7.4				
	$\Delta_r H^\circ$ [KJ mol <sup>-1</sup> ]	$\Delta_r S^\circ$ [J mol <sup>-1</sup> K <sup>-1</sup> ]	$\Delta_r G^\circ$ [KJ mol <sup>-1</sup> ]	$\Delta_r G$ (310 K) [KJ mol <sup>-1</sup> ]	$\Delta_r H^\circ$ [KJ mol <sup>-1</sup> ]	$\Delta_r S^\circ$ [J mol <sup>-1</sup> K <sup>-1</sup> ]	$\Delta_r G^\circ$ [KJ mol <sup>-1</sup> ]	$\Delta_r G$ (310 K) [KJ mol <sup>-1</sup> ]
CA	-30.81 ± 0.35	-65.6 ± 1.2	-11.24 ± 0.11	-10.45 ± 0.18	-26.4 ± 1.5	-67.5 ± 5.0	-6.16 ± 0.11	-5.40 ± 0.06
PA	-21.52 ± 0.22	-51.19 ± 0.73	-6.28 ± 0.06	-5.64 ± 0.06	-	-	-	-
MNG	-52.5 ± 2.0	-124.8 ± 6.6	-15.41 ± 0.23	-13.83 ± 0.30	-46.17 ± 0.26	-117.9 ± 1.2	-11.07 ± 0.57	-9.62 ± 0.59
EC	-44.88 ± 0.06	-105.88 ± 0.21	-13.35 ± 0.17	-12.05 ± 0.24	-45.11 ± 0.62	-106.3 ± 2.1	-13.41 ± 0.17	-12.17 ± 0.06
ECG	-54.0 ± 3.1	-125 ± 10	-16.95 ± 0.17	-15.25 ± 0.24	-51.6 ± 3.6	-117 ± 12	-17.06 ± 0.17	-15.49 ± 0.24
QRC	-60.1 ± 1.5	-133.8 ± 4.9	-20.26 ± 0.34	-18.64 ± 0.30	-70.6 ± 4.2	-172 ± 14	-19.11 ± 0.29	-17.09 ± 0.18
RUT	-62.3 ± 3.0	-144.8 ± 9.9	-19.29 ± 0.80	-17.39 ± 0.30	-69.3 ± 3.3	-170 ± 11	-18.60 ± 0.57	-16.38 ± 0.30
LUT	-72.0 ± 2.8	-171.2 ± 9.5	-21.11 ± 0.40	-18.93 ± 0.36	-77.8 ± 5.5	-195 ± 18	-19.63 ± 0.37	-17.63 ± 0.36
ISR	-54.9 ± 3.6	-126 ± 12	-17.35 ± 0.63	-15.61 ± 0.24	-53.8 ± 3.1	-125 ± 10	-16.43 ± 0.57	-15.14 ± 0.30

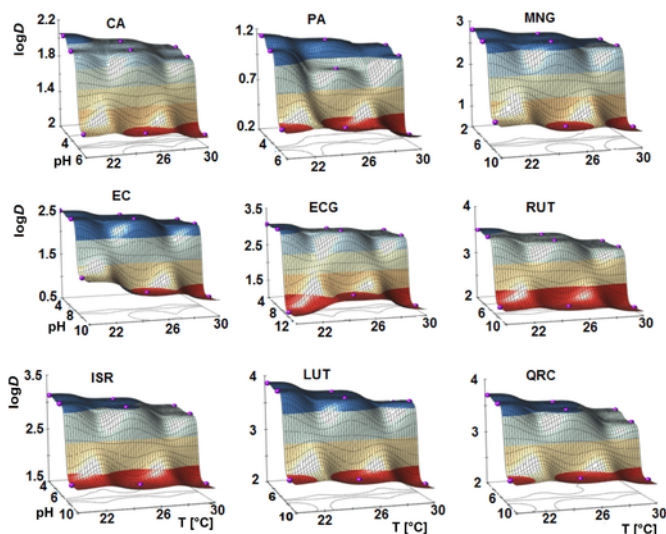


Fig. 6. 3D response surface plots of logD as a function of pH and temperature for *o*-diphenols.

**Table 5**

Quadratic regression models of pH and temperature effect on lipophilicity of *o*-diphenols expressed as logD.

	Model ( $\alpha = 0.05$ )	R <sup>2</sup>	p-Value
CA	$\log D = -42.4360 + 0.0305 \text{ pH} + 0.3134 \text{ T} - 0.1325 \text{ pH}^2 + 0.0027 \text{ pH} * \text{ T} - 0.0006 \text{ T}^2$	0.9988	0.0001
PA	$\log D = 139.751 + 2.5897 \text{ pH} - 0.9471 \text{ T} - 0.3260 \text{ pH}^2 - 0.0016 \text{ pH} * \text{ T} + 0.0015 \text{ T}^2$	0.9925	0.0023
MNG	$\log D = -179.087 + 0.5944 \text{ pH} + 1.241 \text{ T} - 0.0908 \text{ pH}^2 + 0.0005 \text{ pH} * \text{ T} - 0.0021 \text{ T}^2$	0.9991	0.0001
EC	$\log D = 83.005 + 1.6897 \text{ pH} - 0.5431 \text{ T} - 0.0799 \text{ pH}^2 + 0.0026 \text{ pH} * \text{ T} - 0.0008 \text{ T}^2$	0.9992	0.0001
ECG	$\log D = 269.141 + 2.0921 \text{ pH} - 1.7955 - 0.0992 \text{ pH}^2 - 0.003179 \text{ pH} * \text{ T} + 0.00030 \text{ T}^2$	0.9954	0.0011
QRC	$\log D = -94.0789 - 0.5736 \text{ pH} + 0.7016 \text{ T} - 0.0610 \text{ pH}^2 + 0.004 \text{ pH} * \text{ T} - 0.0013 \text{ T}^2$	0.9967	0.0007
RUT	$\log D = -55.5567 - 0.3319 \text{ pH} + 0.4324 \text{ T} - 0.0701 \text{ pH}^2 + 0.0031 \text{ pH} * \text{ T} - 0.0008 \text{ T}^2$	0.9997	0.0000
LUT	$\log D = 25.0250 - 0.7991 \text{ pH} - 0.0958 \text{ T} - 0.0801 \text{ pH}^2 + 0.0050 \text{ pH} * \text{ T} - 0.0002 \text{ T}^2$	0.9995	0.0000
ISR	$\log D = -51.4126 - 0.2452 \text{ pH} + 0.3182 \text{ T} - 0.0689 \text{ pH}^2 + 0.0028 \text{ pH} * \text{ T} - 0.0007 \text{ T}^2$	0.9991	0.0001

to increase the bioavailability of bioactive components, such as phenolic compounds, based on their lipophilic and acidic properties. However, the possibilities of using other HPLC methods, including fast gradient mode and biomimetic columns, for the same research purposes are desired subjects for the future.

Linear solvent strength equations obtained by RP-HPLC under different pH and temperature conditions. Supplementary data to this article can be found online at <https://doi.org/10.1016/j.microc.2018.10.048>.

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For each pH and temperature,  $\log k_w$  was found as intercept of  $\log k = f(\varphi)$  based on LSS Eq. (1). The results of retention factor as a function of methanol percentage in mobile phase obtained at pH 2.8 and 37 °C are shown in Fig. 3A and C. All of the experimental data for

LSS equations obtained under various pH and temperature conditions were summarized in Supplementary material (Table S1). Lipophilicity determination by RP-HPLC-DAD was precise ( $RSD < 0.01$ ) and uncertainty of  $\log k_w$  was  $< 10\%$  at significance level of  $\alpha = 0.32$ .

**Table S1.** Experimental data for linear solvent strength (LSS) equation obtained by RP-HPLC in isocratic elution mode under different pH and temperature conditions

T [°C]	20			25			30			37		
Comp.	φ [%]	Equation	R <sup>2</sup>	φ [%]	Equation	R <sup>2</sup>	φ [%]	Equation	R <sup>2</sup>	φ [%]	Equation	R <sup>2</sup>
<b>pH 2.8</b>												
EC	20-35	y=-.059x+2.19	.999	20-35	y=-.058x+2.06	.999	20-35	y=-.056x+1.93	.999	20-35	y=-.055x+1.75	.999
CA	15-35	y=-.052x+1.79	1.00	15-35	y=-.051x+1.69	1.00	15-35	y=-.051x+1.61	1.00	15-35	y=-.050x+1.48	1.00
MNG	20-35	y=-.064x+2.56	1.00	20-35	y=-.062x+2.42	1.00	20-40	y=-.059x+2.23	1.00	20-40	y=-.059x+2.10	1.00
PA	15-35	y=-.030x+.880	.999	15-35	y=-.030x+.820	.999	15-35	y=-.030x+.756	.999	15-35	y=-.030x+.674	.999
ECG	25-35	y=-.068x+2.81	1.00	20-35	y=-.068x+2.69	1.00	20-40	y=-.065x+2.51	1.00	20-40	y=-.063x+2.30	1.00
RUT	30-50	y=-.062x+3.25	.999	30-50	y=-.062x+3.10	.993	30-50	y=-.059x+2.91	.993	30-50	y=-.056x+2.65	.999
ISR	30-50	y=-.063x+2.88	.999	30-50	y=-.063x+2.77	.996	30-50	y=-.061x+2.59	.996	30-50	y=-.058x+2.35	.999
LUT	40-60	y=-.055x+3.60	.998	40-60	y=-.053x+3.42	.998	40-60	y=-.050x+3.17	.995	45-60	y=-.047x+2.92	.999
QRC	40-60	y=-.054x+3.45	.999	40-60	y=-.052x+3.27	.999	40-55	y=-.050x+3.08	.995	40-60	y=-.048x+2.86	.999
<b>pH 4.0</b>												
EC	20-40	y=-.058x+2.18	.999	20-40	y=-.057x+2.05	.999	20-40	y=-.055x+1.91	.999	15-35	y=-.058x+1.85	.999
CA	25-40	y=-.026x+1.67	.990	20-35	y=-.041x+1.66	.994	15-30	y=-.035x+1.56	1.00	10-35	y=-.036x+1.55	.990
MNG	25-40	y=-.061x+2.50	1.00	20-35	y=-.059x+2.36	.990	20-35	y=-.061x+2.31	1.00	10-35	y=-.053x+1.91	.990
PA	20-40	y=-.019x+.783	.999	25-40	y=-.020x+.597	.995	15-30	y=-.024x+.697	.999	10-35	y=-.025x+.663	.999
ECG	25-40	y=-.068x+2.79	.999	25-40	y=-.066x+2.62	.999	20-40	y=-.066x+2.52	.999	20-35	y=-.065x+2.37	.999
RUT	35-45	y=-.063x+3.27	.999	30-45	y=-.062x+3.12	.999	27-45	y=-.062x+3.01	.999	25-45	y=-.061x+2.83	.996
ISR	30-45	y=-.063x+2.94	.999	27-45	y=-.065x+2.82	.999	27-45	y=-.063x+2.68	.999	25-45	y=-.064x+2.59	.995
LUT	40-60	y=-.054x+3.61	.998	40-60	y=-.052x+3.41	.998	40-60	y=-.051x+3.25	.998	40-60	y=-.049x+3.00	.999
QRC	-	-	-	40-60	y=-.052x+3.25	.999	40-60	y=-.050x+3.10	.999	40-60	y=-.048x+2.86	.999
<b>pH 5.7</b>												
EC	20-35	y=-.058x+2.14	.998	20-35	y=-.058x+2.06	.999	20-35	y=-.056x+1.93	.999	15-35	y=-.063x+2.14	.998
CA	2-12	y=-.05x+.931	1.00	2-15	y=-.055x+.890	1.00	2-15	y=-.056x+.834	1.00	5-15	y=-.054x+.726	1.00
MNG	20-35	y=-.063x+2.44	1.00	20-35	y=-.063x+2.73	1.00	20-35	y=-.062x+2.22	1.00	15-35	y=-.057x+1.83	1.00
PA (pH 5)	-	-	-	10-25	y=-.014x+.092	.997	-	-	-	-	-	-
ECG	20-40	y=-.068x+2.78	1.00	20-35	y=-.068x+2.72	1.00	20-35	y=-.066x+2.55	1.00	15-35	y=-.067x+2.41	1.00
RUT	30-50	y=-.062x+3.22	.999	30-50	y=-.062x+3.09	.999	30-50	y=-.060x+2.92	.997	30-45	y=-.059x+2.72	.999
ISR	30-50	y=-.063x+2.85	.999	30-50	y=-.063x+2.73	.999	30-50	y=-.061x+2.56	.997	30-45	y=-.060x+2.40	.998
LUT	40-60	y=-.055x+3.57	.999	40-60	y=-.053x+3.40	.997	40-60	y=-.053x+3.31	.999	40-60	y=-.049x+3.00	.999
QRC	40-60	y=-.053x+3.41	.999	40-60	y=-.052x+3.24	.998	40-60	y=-.048x+2.98	.995	40-60	y=-.048x+2.86	.999
<b>pH 7.4</b>												
EC	20-35	y=-.059x+2.21	.999	20-35	y=-.058x+2.07	.999	20-35	y=-.057x+1.94	.999	20-35	y=-.055x+1.77	.999
CA	5-15	y=-.052x+.899	1.00	5-12	y=-.052x+.805	1.00	5-15	y=-.050x+.749	1.00	10-20	y=-.050x+.635	1.00
MNG	15-35	y=-.060x+1.79	1.00	15-35	y=-.057x+1.67	.990	15-35	y=-.050x+1.52	1.00	15-35	y=-.046x+1.34	1.00
ECG	20-35	y=-.069x+2.82	1.00	20-35	y=-.069x+2.71	1.00	20-35	y=-.066x+2.53	1.00	20-35	y=-.064x+2.33	1.00
RUT	35-50	y=-.062x+3.16	.999	30-40	y=-.061x+2.98	.996	25-40	y=-.059x+2.79	.999	30-45	y=-.059x+2.49	.999
ISR	35-50	y=-.063x+2.80	.999	40-55	y=-.061x+2.61	.995	25-45	y=-.060x+2.45	.999	25-45	y=-.059x+2.27	.999
LUT	45-60	y=-.053x+3.47	.999	40-55	y=-.051x+3.16	.999	40-60	y=-.048x+2.96	.993	45-60	y=-.046x+2.69	.999
QRC	45-60	y=-.053x+3.31	.999	40-55	y=-.050x+3.07	.999	40-55	y=-.049x+2.92	.992	45-60	y=-.046x+2.60	.999
<b>pH 8.6</b>												
EC	20-35	y=-.057x+2.04	.999	20-35	y=-.055x+1.91	.999	20-35	y=-.055x+1.83	.999	15-35	y=-.057x+1.73	.999
CA	7-12	y=-.050x+.399	1.00	7-15	y=-.052x+.420	.990	7-20	y=-.043x+.321	1.00	7-20	y=-.045x+.304	1.00
MNG	20-35	y=-.059x+.861	1.00	20-35	y=-.058x+.808	1.00	20-35	y=-.046x+.574	1.00	15-35	y=-.058x+.730	1.00
ECG	20-35	y=-.065x+2.48	1.00	20-35	y=-.062x+2.32	1.00	20-35	y=-.061x+2.20	1.00	15-35	y=-.061x+2.08	1.00
RUT	25-40	y=-.052x+1.96	.999	25-40	y=-.051x+1.90	.999	25-40	y=-.050x+1.80	.999	25-45	y=-.048x+1.67	.999
ISR	25-40	y=-.050x+1.59	.999	25-40	y=-.049x+1.52	.999	25-40	y=-.049x+1.46	.999	25-45	y=-.031x+.885	.999
LUT	35-55	y=-.040x+2.18	.999	35-55	y=-.039x+2.11	.999	35-55	y=-.038x+2.04	.999	35-55	y=-.037x+1.95	.999
QRC	35-50	y=-.040x+2.19	.999	35-50	y=-.040x+2.15	.999	35-50	y=-.039x+2.03	.999	35-50	y=-.038x+1.97	.999
<b>pH 10.2</b>												
EC	10-25	y=-.066x+.945	.985	10-25	y=-.043x+.672	.997	10-25	y=-.037x+.512	.991	-	-	-
MNG	-	-	-	2-10	y=-.099x+.004	1.00	-	-	-	-	-	-
ECG	10-20	y=-.044x+.850	1.00	10-25	y=-.047x+.849	1.00	10-25	y=-.047x+.837	1.00	-	-	-
RUT	15-25	y=-.052x+.954	.999	15-30	y=-.084x+1.40	.999	-	-	-	-	-	-
ISR	15-25	y=-.047x+.429	.980	15-30	y=-.083x+.872	.999	-	-	-	-	-	-
LUT	30-50	y=-.047x+1.30	.999	30-45	y=-.048x+1.27	.999	-	-	-	-	-	-
QRC	-	-	-	30-40	y=-.039x+.776	.999	-	-	-	-	-	-

CA – chlorogenic acid; EC – epicatechin; ECG – epicatechin gallate; ISR – isoorientin; LUT – luteolin; MNG – mangiferin; PA – protocatechuic acid; QRC – quercetin; RUT – rutin; φ – percentage of organic modifier (methanol)