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Evaluation of Antioxidative Activity of Croatian Propolis Samples Using DPPH· and ABTS·+ Stable Free Radical Assays

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Abstract: Propolis is one of the richest sources of plant phenolics (flavonoids and phenolic acids), which are widely recognized as rather strong antioxidants. The aim of our work was to use colored stable free radical (DPPH· and ABTS·+) spectrophotometric and thin-layer chromatographic (TLC) assays to study the antioxidative behavior of the phenolics (caffeic acid, galangin and pinocembrin) most commonly present in Croatian propolis samples obtained from different Croatian regions. We propose a mathematical model providing a more sophisticated interpretation of the obtained results and a new parameter named antioxidative efficiency (*AOE*) is introduced. The kinetic behaviour of chosen standards determined by spectrophotometric assays follows the exponential decrease of the absorption curve. Explained numerically, *AOE* represents the absolute value of the first derivative of an absorbance curve in the point A_0/e (where A_0 is the absorbance measured at t = 0 and e is the natural logarithm base). The advantage of this newly introduced parameter is that it provides an easy and accurate mutual comparison between the rates of antioxidative efficiency of different propolis samples. A TLC assay was only applicable in the case of the DPPH· radical. Dose-response curves were described using a linear function

with *AOE* expressed as a coefficient of the slope. The chromatographic method was shown to be very rapid, reliable and easy-to-perform.

1. Introduction

Propolis is a resinous hive product collected by honeybees (*Apis melifera*, L.) from various plant sources. Bees use it as a construction material, to smooth internal walls of the hive and preserve it from extreme moisture and drought conditions. They also use it to embalm dead invaders and in such way, prevent the development and spread of microbial diseases. Propolis is widely used in traditional medicine and is reported to have a broad spectrum of pharmacological activities. Besides its traditional uses, it has recently gained popularity as a food supplement in numerous countries, claimed to improve health and prevent diseases. Numerous contemporary *in vitro* studies have proven its antioxidative, antiimflamatory, immunomodulatory and antimicrobial activities [1,2].

Recently there has been increased interest in the role of reactive oxygen species in biological systems and their implied roles in various pathological states. Accordingly, attention is being focused on the protective biochemical functions of naturally occurring antioxidants. Propolis is one of the richest sources of the plant phenolics (flavonoids and phenolic acids), which are generally known as rather strong antioxidants [3-6]. Since the composition of propolis varies with its origin (it depends primarily upon the vegetation of the area from where it was collected), the intensity of antioxidative activity should be variable as well. Several methods have been proposed to measure the free radical scavenging capacity (RSC), regardless of the individual compounds which contribute to the total free radical scavenging capacity of a plant product. Each method relies to the generation/scavenging of a different radical, acting through a variety of mechanisms, and measurement of a range of end points either at a fixed time point varying the concentration of antioxidant or over a certain time range. For the comparison of different plant extracts easy, rapid and reliable methods can be very useful, such as measuring the disappearance of colored stable free radicals such as the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS$^{\cdot+}$) and the 2,2-diphenyl-1-picryl-hydrazyl (DPPH$^{\cdot}$) radical [7-14]. The aim of the work presented in this paper was to study the kinetic behavior of the most phenolics commonly present in Croatian propolis samples from different Croatian regions by using above mentioned free radical assays. In the manuscript we propose a mathematical model for providing a more sophisticated interpretation of the obtained results as well. According to our previous findings [15,16], we chose caffeic acid as a compound representative of phenolic acids, while galangin and pinocembrin were used to represent flavones and flavanones, respectively. The mixture of these three standards was used to develop a mathematical model for determination of antioxidative behavior of tested propolis extracts. Also, we have developed a thin-layer chromatography (TLC) method as an alternative to the already existing spectrophotometric assays. Separation, identification and quantification of the commonly present flavonoids and phenolic acids in the chosen propolis samples were performed using HPLC with photodiode array detection (DAD), which enables the identification of compounds not only by their retention times, but also their individual spectra.

2. Results and Discussion

2.1. HPLC analysis

Composition analysis was performed by using HPLC with UV-Vis detection, whereby compounds in the propolis samples were identified by comparing their retention times and UV spectra with those of standard compounds. Figure 1 presents the structures of investigated phenolics, while Table 1 lists the phenolic compound contents in different propolis samples.

Figure 1. Structures of phenolic acids and flavonoids found in Croatian propolis samples.
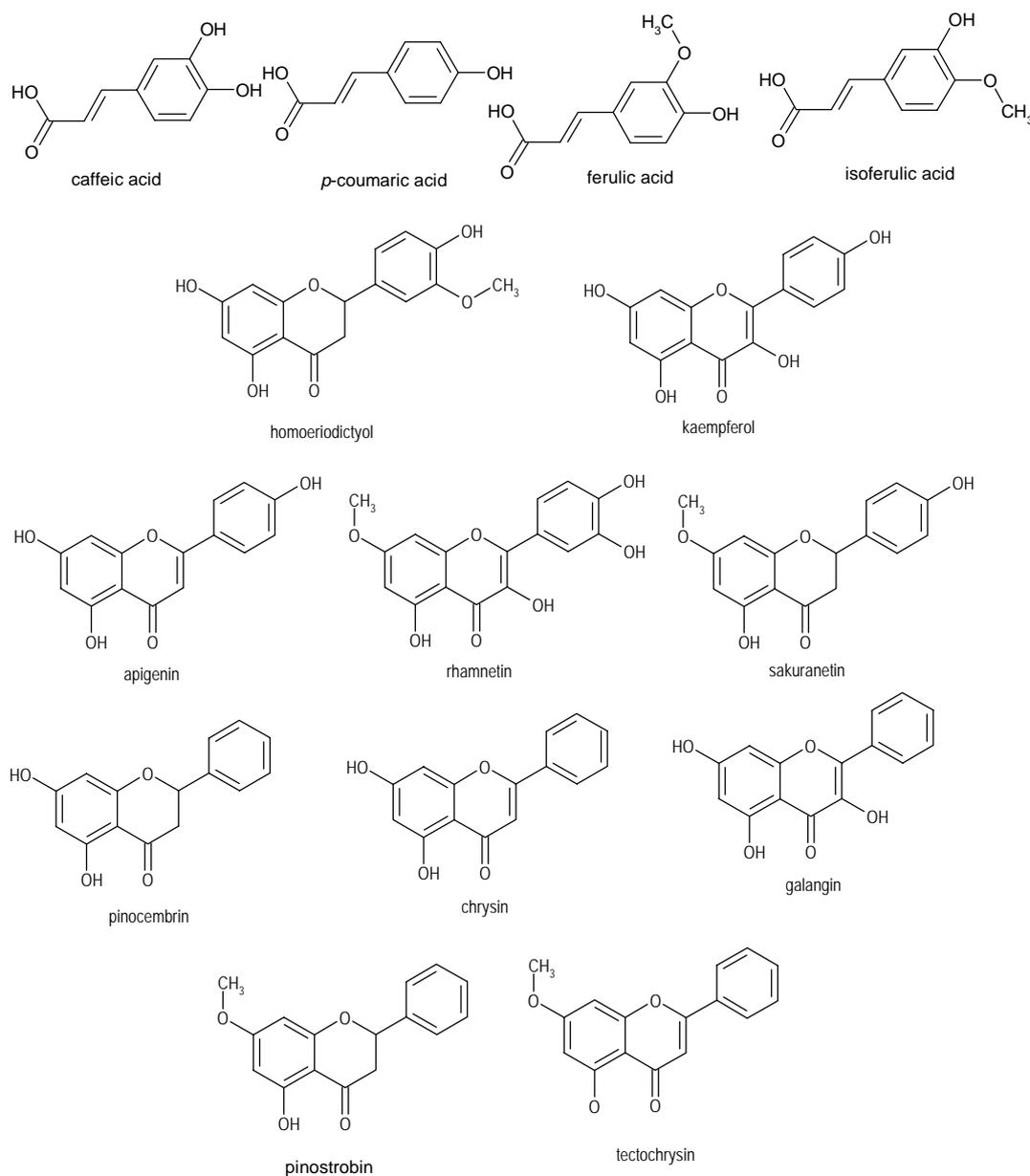
Table 1. Content of the constituents in investigated ethanolic propolis extracts.

Standard compound	Metković	Čisla (Omiš)	Pelješac	Vis	Kutina
	PROPOLIS SAMPLE				
	c (µg/mL) ± S.D.[*]				
Caffeic acid	6.45 ± 0.07	7.25 ± 0.07	7.23 ± 0.07	5.69 ± 0.07	2.39 ± 0.03
p-Coumaric acid	4.66 ± 0.04	23.91 ± 0.19	7.79 ± 0.07	18.01 ± 0.11	1.29 ± 0.02
Ferulic acid	1.92 ± 0.02	25.28 ± 0.21	7.96 ± 0.07	–	1.20 ± 0.01
Isoferulic acid	4.64 ± 0.04	3.45 ± 0.03	3.35 ± 0.03	4.27 ± 0.04	1.08 ± 0.01
Homoeridictyol	10.30 ± 0.11	3.14 ± 0.03	6.01 ± 0.06	2.65 ± 0.02	9.81 ± 0.09
Kaempferol	6.90 ± 0.07	4.94 ± 0.05	5.40 ± 0.06	4.54 ± 0.05	–
Apigenin	5.39 ± 0.05	3.26 ± 0.03	2.44 ± 0.02	2.71 ± 0.02	–
Rhamnetin	2.43 ± 0.02	–	2.45 ± 0.02	1.36 ± 0.02	–
Sakuranetin	12.51 ± 0.11	–	5.72 ± 0.05	2.52 ± 0.02	–
Pinocembrin	25.59 ± 0.22	13.36 ± 0.10	19.83 ± 0.10	13.21 ± 0.11	4.52 ± 0.05
Chrysin	98.13 ± 0.55	13.62 ± 0.12	71.54 ± 0.07	13.31 ± 0.14	42.22 ± 0.40
Galangin	20.42 ± 0.20	6.85 ± 0.06	16.67 ± 0.17	4.76 ± 0.04	1.79 ± 0.02
Pinostrobin	27.50 ± 0.25	–	16.30 ± 0.16	–	7.18 ± 0.06
Tectochrysin	31.42 ± 0.30	–	18.73 ± 0. 18	–	10.96 ± 0.10
Total amount of identified flavonoids (µg/mL)	240.59	45.17	165.09	45.06	76.48
Total amount of identified phenolic acids (µg/mL)	17.67	59.89	26.33	27.97	5.96

[*] Values are expressed as a mean value of triplicate analyses for each sample ± standard deviation (S.D.).

– Not detected; number of unidentified peaks found in propolis samples: 20 (Metković), 16 (Omiš), 22 (Pelješac), 18 (Vis), 18 (Kutina)

Out of 21 chosen standards, 14 or less were identified and quantified. Also, in every propolis sample we have found a certain number of phenolics that were not identified, but the peak areas of those compounds were significantly lower than the areas of identified peaks (data not shown). Figures 2 and 3 show the chromatograms obtained after the analysis of the propolis samples from Pelješac and Kutina, respectively. The propolis sample from Čisla (Omiš) had the highest amount of phenolic acids, while the samples from Metković and Pelješac had the most diverse composition, and the highest amount of flavones (such as chrysin and tectochrysin). The propolis sample collected around Kutina contained the smallest amount of phenolics.

Figure 2. HPLC chromatogram of the propolis sample from Pelješac, recorded at 270 nm.
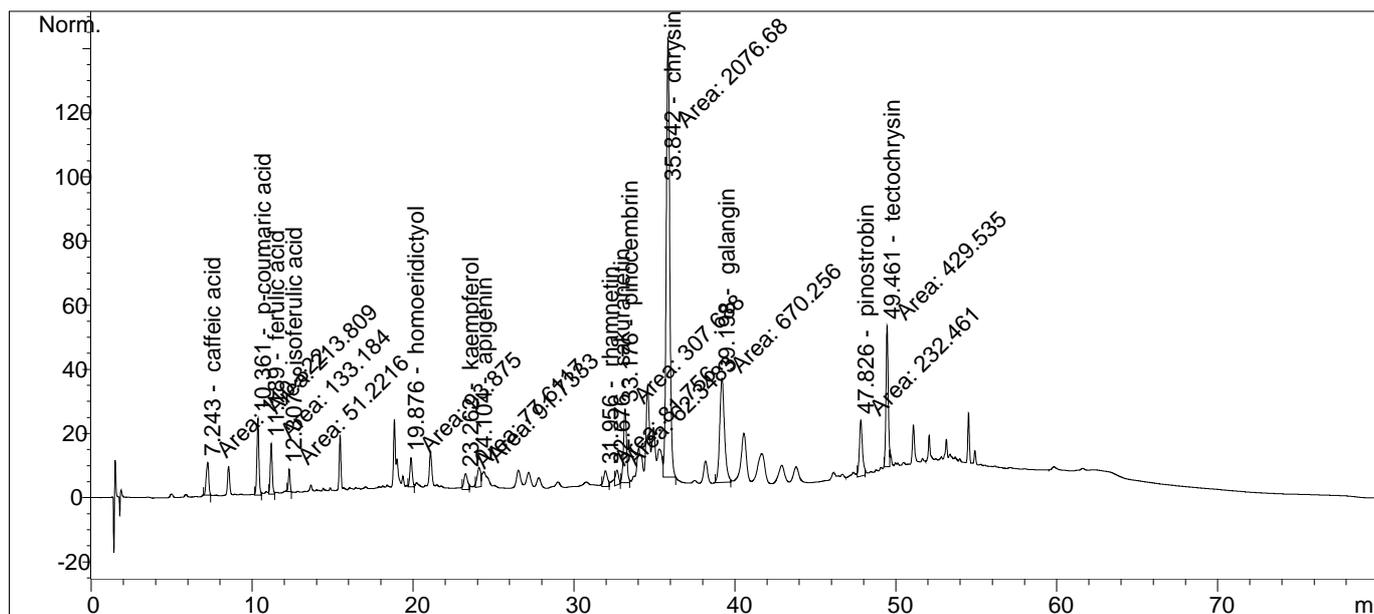
Figure 3. HPLC chromatogram of the propolis sample from Kutina, recorded at 270 nm.
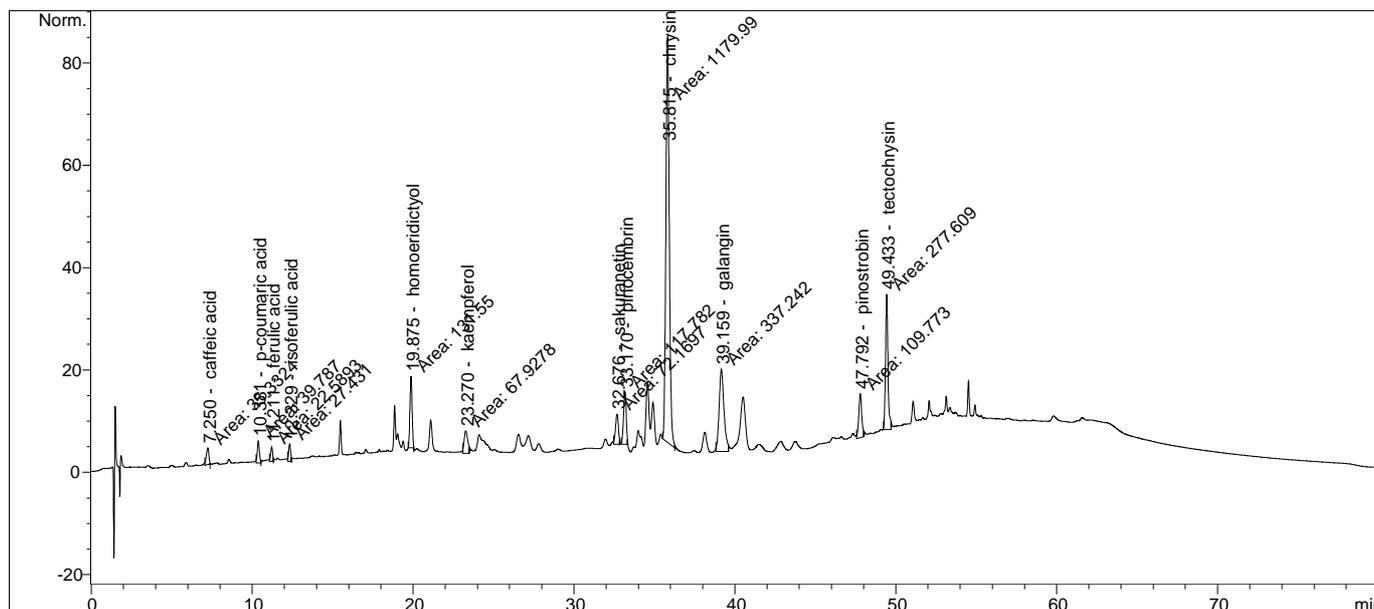
2.2. Spectrophotometric and thin-layer chromatographic (TLC) assays

In the last few decades free radicals and antioxidants have been the subject of numerous *in vitro* and *in vivo* studies. Antioxidative activity has become the one of the most interesting biological properties of naturally occurring substances present in higher plants. Beneficial health effects of an array of herbal preparations have been ascribed to the ability of plant extracts to scavenge free radicals, and in such way as to prevent the development of various diseases (e.g. rheumatoid arthritis, diabetes, Alzheimer's and Parkinson's disease) [17,18]. Because of the complex nature of phytochemicals, an

easy way to evaluate the antioxidative activity of different extracts is to measure a total antioxidative efficiency using spectrophotometric or TLC stable free radical (DPPH$^{\cdot}$ or ABTS$^{\cdot+}$) decolorization assays. Results obtained in such a way could encourage further, more complex *in vitro* and *in vivo* studies of antioxidative activity.

The most common parameter for expressing the antioxidative activity of a pure compound or different plant extracts by DPPH$^{\cdot}$ assay is the *effective concentration* (*EC$_{50}$*), which represents the concentration of analyzed sample sufficient to obtain 50% of a maximum scavenging capacity (measured as the 50% decrease in DPPH$^{\cdot}$ absorbance from its initial value) [9,12]. Since this parameter does not take into account the rate of absorbance decrease, a time-dependent parameter is introduced as a correction factor (*T$_{EC50}$* – time point in which radical absorbance is decreased to 50%). Taking into account that both parameters (*EC$_{50}$* and *T$_{EC50}$*) affect the antiradical capacity, a new parameter called *antiradical efficiency* (*AE*) was introduced by Sánchez-Moreno *et al*. [8]:

$$AE = 1/\ EC_{50} \ \text{x} \ T_{EC_{50}} \tag{1}$$

Re *et al*. [10] introduced in their work the notion of a relative measure for antioxidative activity of the tested compounds. Total contribution to the antioxidant activity over the time range was studied by calculating the area under the curve (AUC), derived from plotting the gradient of the percentage inhibition/concentration plots as a function of time reaction. In this way, the ratio between the AUC for the reaction of the specific antioxidant and for Trolox$^{®}$ (a water soluble equivalent of vitamin E) represents the measure for antioxidant activity, and it is a relative one.

In the present work we used DPPH$^{\cdot}$ radical and ABTS$^{\cdot+}$ radical cation spectrophotometric assays, together with a thin-layer chromatography (TLC) method, to establish the kinetics of the antioxidative behavior of the most commonly present phenolics in Croatian propolis samples (caffeic acid, galangin and pinocembrin) and also to describe the antioxidative behavior of investigated propolis extracts. Using the mixture of listed standards, we developed the appropriate mathematical models for providing more sophisticated interpretation of antioxidative activity. In our work, we introduce a new parameter called antioxidative efficiency (*AOE*), which enables an easy and mutual comparison between rates of antioxidative activities of different propolis samples.

2.3. Spectrophotometric DPPH$^{\cdot}$ assay

Preliminary results demonstrated a time-dependency of the reaction, and the greatest difference in the decrease of absorbance at 518 nm (as a function of different standard mixture concentrations) was observed after 30 seconds (data not shown). Therefore, that time point was chosen to measure the extent of decrease of the DPPH$^{\cdot}$ radical absorbance, plotted as a function of standard mixture concentration (Figure 4).

The dose-response curve indicates a biphasic reaction, with a very rapid initial phase followed by a slower phase that asymptotically approaches certain value close to zero, and can be generally described with the equation:

$$A = A_0 e^{-Bc} + y_0, \tag{2}$$

where *A* represents the absorbance of the free radical measured after 30 s, A_0 is the absorbance measured at $t = 0$, *c* is the concentration of the standard mixture, while *B* and y_0 are constants which

represent the exponential decrease of the curve and horizontal asymptote to which values of absorbance are approaching.

Figure 4. Decrease in absorbance of DPPH$^{.}$ radical at 518 nm, measured after 30 seconds, depending on the concentration of standard solution (a mixture of caffeic acid, galangin and pinocembrin).
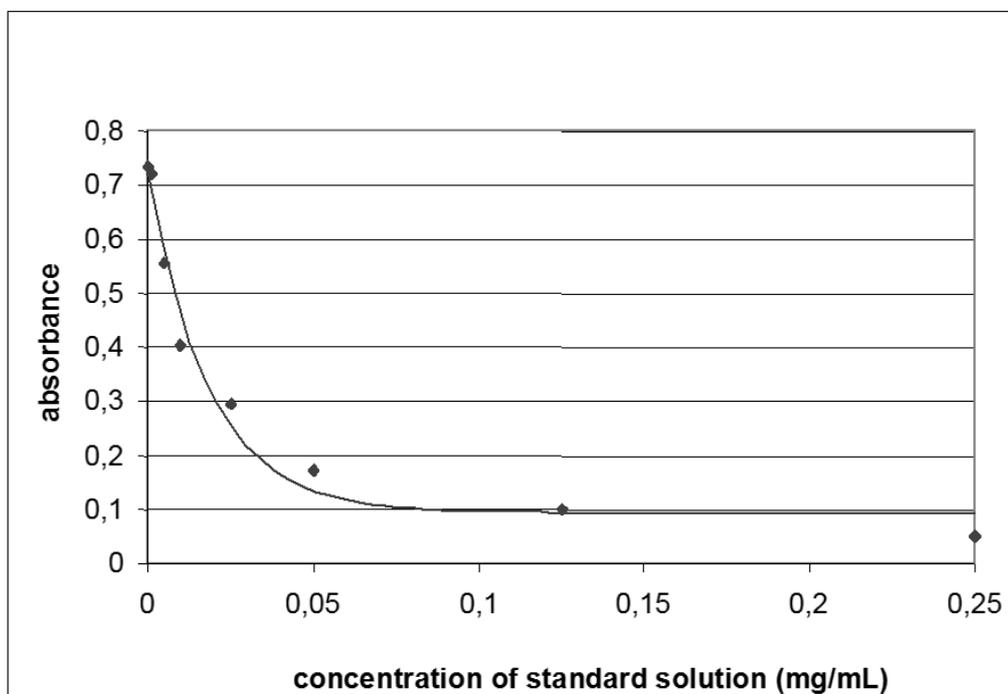
$$A = 0.632e^{-54.259c} + 0.093$$
$$n = 8, \ r^2 = 0.982 \ .$$

In order to determine the antioxidative activity and to mutually compare different propolis samples, we introduce the parameter called *antioxidative efficiency* (*AOE*), defined as an absolute value of the first derivative of the curve at the point with an absorbance value of A_0/e. Geometrically, *AOE* represents the absolute value of the slope of the tangent on the absorption curve at the point A_0/e. In this way samples with greater exponential decrease in free radical absorbance (and related narrower slope of the tangent in the same point) have higher *AOE* values. The general concentration-response curve with the exponential decrease of the absorbance and the tangent on the absorbance-concentration curve in the point A_0/e is shown in Figure 5.

Antioxidative efficiency as the first derivation of the absorbance-concentration curve can be calculated according to the expression:

$$a = -A_0 Be^{-Bc} \ . \tag{3}$$

The calculated equation of the tangent on the absorbance-concentration curve at 518 nm is:

$$y = -9.567x + 0.495 , \tag{4}$$

giving the value 9.567 for *AOE*.

Using the same model, we evaluated the antioxidative efficiency of propolis samples from different Croatian regions. Table 2 presents equations describing the decrease in DPPH˙ absorbance as a function of propolis extract concentrations, together with *AOE* parameters.

Figure 5. General concentration-response curve ($A = A_0 e^{-Bc} + y_0$), with the characteristic parameters defining *AOE*.
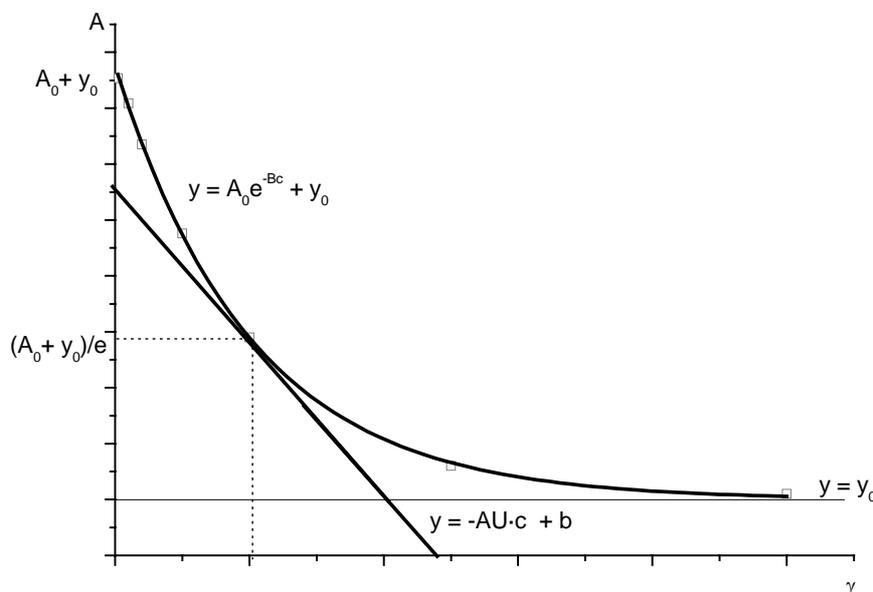

Table 2. Antioxidative efficiency of Croatian propolis samples determined by spectro-photometric DPPH˙ assay.

Propolis sample	Curve equation	Correlation coefficient	Equation of the tangent $A = A_0 / e$	Antioxidative efficiency (*AOE*)
Metković	$A = 0.801 e^{-10.955c} + 0.075$	$r^2 = 0.99796$	$y = -2.679x + 0.612$	2.679
Čisla (Omiš)	$A = 0.776 e^{-10.480c} + 0.075$	$r^2 = 0.99846$	$y = -2.352x + 0.599$	2.352
Pelješac	$A = 0.711 e^{-12.390c} + 0.123$	$r^2 = 0.99766$	$y = -2.267x + 0.554$	2.267
Island Vis	$A = 0.744 e^{-12.117c} + 0.153$	$r^2 = 0.99239$	$y = -2.164x + 0.586$	2.164
Kutina	$A = 0.876 e^{-2.779c} + 0.000$	$r^2 = 0.97530$	$y = -0.880x + 0.639$	0.880

2.4. Spectrophotometric ABTS˙+ assay

Following the same principle as for the DPPH˙ assay, we determined the time point to measure the decrease in ABTS˙+ absorbance (60 s). Using standard mixture solutions, we established the appropriate mathematical model for this assay as well. This reaction is also biphasic, with a very rapid initial phase, followed by a slower phase that, as the solution becomes colorless, approaches a zero absorbance value (Figure 6).

Figure 6. Decrease in the absorbance of ABTS$^{·+}$ radical cation at 730 nm, measured after 60 seconds, depending on the concentration of standard solution (a mixture of caffeic acid, galangin and pinocembrin).
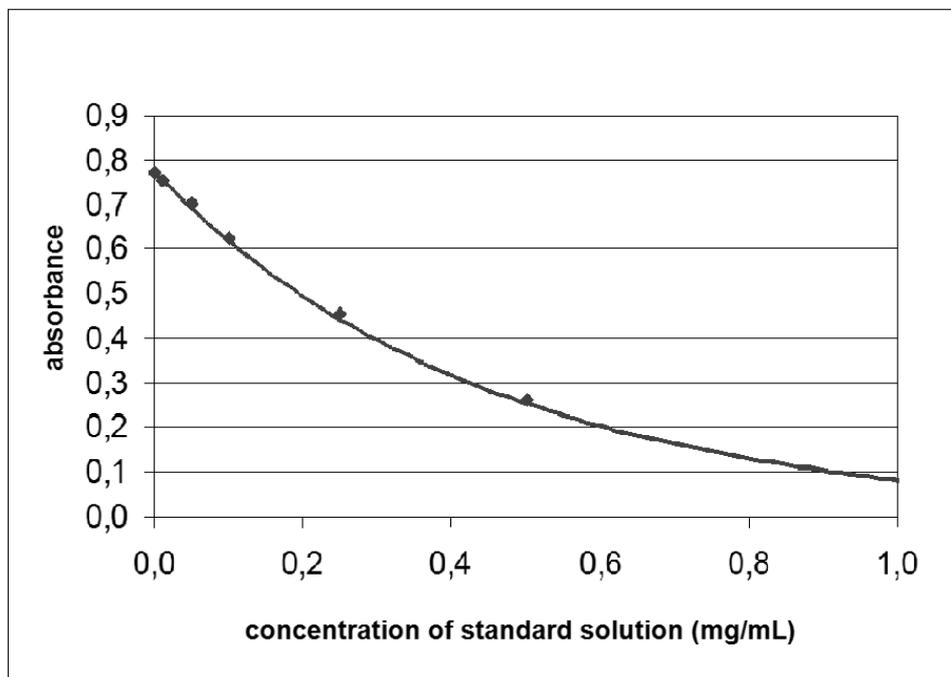
$$A = 0.773e^{-2.226\gamma}$$
$$n = 7, \ r^2 = 0.999.$$

Such a function can be generally described with the expression:

$$A = A_0e^{-Bc}, \tag{5}$$

where *A* represents the absorbance of the free radical measured after 60 seconds, A_0 the absorbance measured at *t* = 0, *c* concentration of the standard mixture, while *B* is a constant, describing the exponential drop of the curve.

Antioxidative efficiency (*AOE*), according to the DPPH$^{·}$ model, is equal to the absolute value of the first derivative of the curve at the point with absorbance value A_0/e, and in this case it is described with the equation of the tangent:

$$y = -0.633x + 0.569 \tag{6}$$

with *AOE* equal to 0.633.

According to the model, we evaluated the antioxidative efficiency of used propolis samples, with the results shown in Table 3.

2.5. Thin-layer chromatography

In the present paper, besides spectophotometric assays, we also evaluated the applicability of thin-layer chromatograpy methods for determination of antioxidative activity. The DPPH$^{·}$ procedure was selected as the appropriate assay, since the radical was more stable on the support layer and the coloration of sample bands was more homogenous in comparison to one obtained with ABTS$^{·+}$ radical

cation. Results achieved using the latter radical as a spraying agent were not reproducible, and dose-dependent antioxidative activity could not be precisely determined (data not shown).

Table 3. Antioxidative efficiency of Croatian propolis samples determined by spectro-photometric ABTS[·+] assay.

Propolis sample	Curve equation	Correlation coefficient	Equation of the tangent $A = A_0/e$	Antioxidative efficiency (*AOE*)
Island Vis	$A = 0.701e^{-0.582c}$	$r^2 = 0.9937$	$y = -0.150x + 0.516$	0.150
Čisla (Omiš)	$A = 0.838e^{-0.417c}$	$r^2 = 0.9808$	$y = -0.129x + 0.617$	0.129
Pelješac	$A = 0.825e^{-0.395c}$	$r^2 = 0.9949$	$y = -0.120x + 0.607$	0.120
Metković	$A = 0.701e^{-0.407c}$	$r^2 = 0.9944$	$y = -0.105x + 0.516$	0.105
Kutina	$A = 0.893e^{-0.101c}$	$r^2 = 0.9804$	$y = -0.033x + 0.657$	0.033

Using scanning densitometry, we determined the characteristic values that describe chromatographic peaks - peak height (*H*) and area (*A*). Analyzing the antioxidative efficiency of standard mixture, we established linear correlation between the decrease of DPPH[·] absorbance (expressed as peak height), and concentration of standards. Figure 7 shows the chromatogram, while Figure 8 presents linear dose-response curve of decrease in DPPH[·] radical absorbance as a function of antioxidants' concentration.

Figure 7. Chromatogram obtained after spraying the layer (applied standard mixture bands in the range from 45 μg/mL – track 1 to 10 μg/mL – track 8) with 0.3 mM solution of DPPH[·] radical.
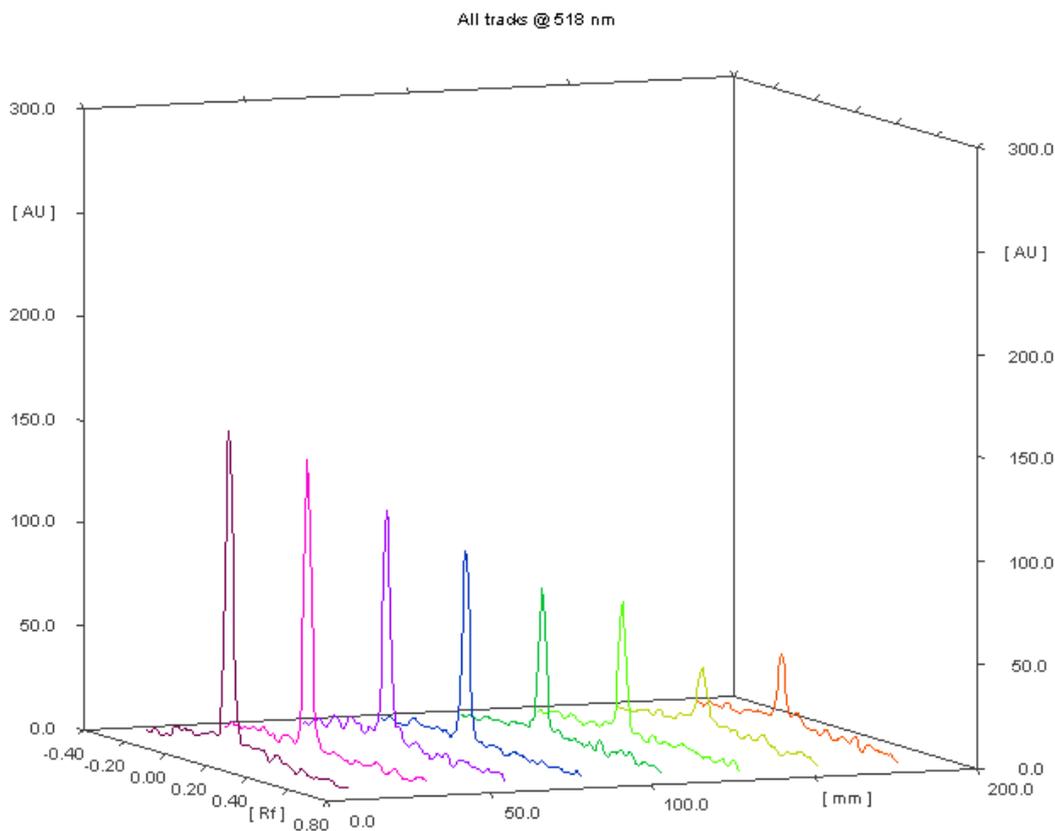
The absolute value of the slope of the tangent on the absorbance curve was taken as a measure of antioxidative efficiency of standard solution, and it is equal to 1.895. Propolis extracts also showed a linear dose-dependent behavior, and parameters that describe their antioxidative efficiency are presented in Table 4.

For the TLC assay, applied concentrations of standard solution and propolis extracts were in a more narrow range then those used for spectrophotometric assays, and dose-response curves are described using a linear function with antioxidative efficiency expressed as a coefficient of the slope. The band-blot DPPH˙ test was shown to be very rapid, reliable and easy-to-perform, and can be used as an alternative method to the spectrophotometric ones.

Figure 8. Linear dose-response curve of decrease in DPPH˙ radical absorbance (expressed as peak height in absorbance units – *AU*) as a function of standard mixture concentration.
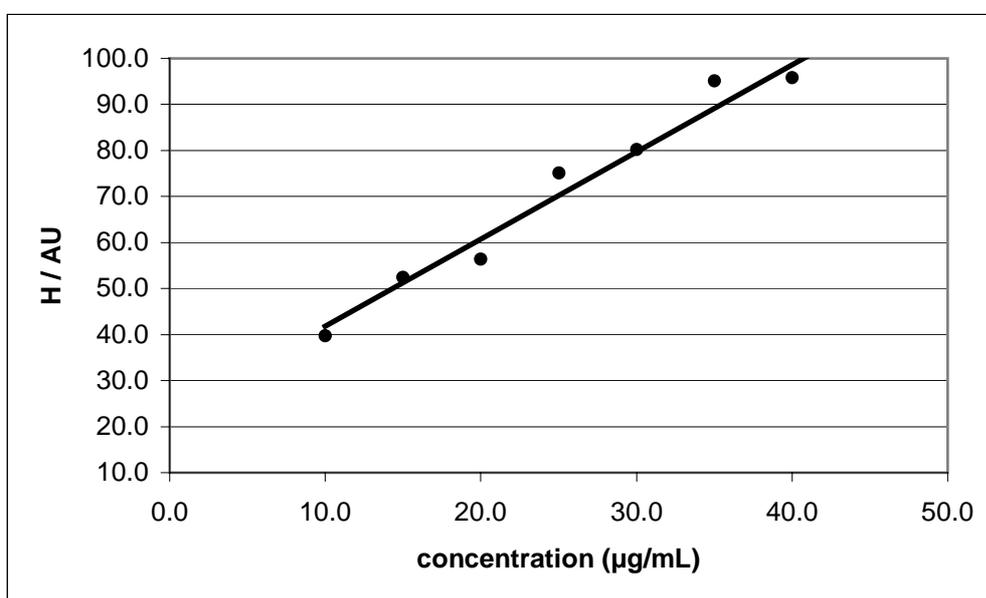
$$H = 1.89c + 22.856$$
$$n = 7, \ r^2 = 0.974.$$

Table 4. Antioxidative efficiency of Croatian propolis samples determined by TLC-DPPH˙ assay.

Propolis sample	Curve equation	Correlation coefficient	Antioxidative efficiency (*AOE*)
Pelješac	$y = 0.452x + 25.441$	$r^2 = 0.9876$	0.452
Čisla (Omiš)	$y = 0.391x + 23.877$	$r^2 = 0.9900$	0.391
Metković	$y = 0.389x + 40.464$	$r^2 = 0.9756$	0.389
Island Vis	$y = 0.327x + 19.249$	$r^2 = 0.9751$	0.327
Kutina	$y = 0.202x - 2.045$	$r^2 = 0.9862$	0.202

Considering the evaluation of the antioxidative efficiency, we noticed some discrepancies between results obtained by using DPPH· and ABTS·+ radical cation spectrophotometric assays and the DPPH· band-blot test. With each assay a bit different arrangement of tested samples (from the sample with the highest activity to the one with the lowest activity) was obtained. According to our experience, by using described methods it was possible to get the information about the relative antioxidative efficiency, meaning that given assays differ between the group of samples with the high antioxidative activity and those with the low activity. To mutually compare different samples it is advisable to use only one of describe assays but to make a final conclusion about total antioxidative efficiency of the sample, other methods described in literature should be used as well. In order to evaluate the applicability of the assays used in our investigatrion, we established the correlation between the concentration of present flavonoids and phenolic acids in analyzed propolis samples and determination of antioxidative efficiency by using multiple regression analysis and we obtained the following regression equations:

1. for the spectrophotometric DPPH· assay:

$$AOE = 0.5637 + 0.0285\ c_{\text{(phenolic acids)}} + 0.0063\ c_{\text{(flavonoids)}} \tag{7}$$
$$n = 5,\ r = 0.878,\ s = 0.4713,\ F = 3.3623,$$

2. for the spectrophotometric ABTS·+ assay:

$$AOE = 0.0499 + 0.0016\ c_{\text{(phenolic acids)}} + 0.0001\ c_{\text{(flavonoids)}} \tag{8}$$
$$n = 5,\ r = 0.678,\ s = 0.0465,\ F = 0.8494 \text{ and}$$

3. for the band-blot DPPH· assay:

$$AOE = 0.1449 + 0.0040\ c_{\text{(phenolic acids)}} + 0.0009\ c_{\text{(flavonoids)}} \tag{9}$$
$$n = 5,\ r = 0.885,\ s = 0.0624,\ F = 3.6317.$$

According to the above given equations and statistical parameters, together with larger stability and solubility of DPPH· radical and better reproducibility of obtained results as well, our conclusion would be that DPPH· radical should be used for the analysis of total antioxidative capacity.

3. Experimental

3.1. Chemicals

Propolis samples were obtained from different Croatian regions - Metković, Čisla (Omiš), Vis island, Pelješac and Kutina. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·) were purchased from Sigma-Aldrich (Steinheim, Germany). Potassium persulfate ($K_2S_2O_8$) together with methanol (gradient grade for liquid chromatography) and formic acid 98-100% were obtained from Merck (Darmstadt, Germany), while 96% ethanol (p.a.) was purchased from Kemika (Zagreb, Croatia). The phenolic acids caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid and ammonium formate p.p.a. for mass spectroscopy were obtained from Fluka (Buchs, Switzerland), while the flavonoids quercetin, homoeridictyol, genistein,

kaempferol, apigenin, rhamnetin, sakuranetin, isosakuranetin, chrysin, acacetin, galangin, pinostrobin, naringenin, tamarixetin, pinocembrin, chrysin-dimethylether, apigenin-trimethylether were purchased from Extrasynthese (Genay Cedex, France).

3.2. HPLC analysis

The HPLC analyses were carried out on an Agilent 1100 HPLC system equipped with a diode-array detector controlled by ChemStation for LC 3D software (Agilent Technologies, USA). For the separation of flavonoids and phenolic acids, an X-bridge C_{18} column (150 x 3.0 mm, 3.5 μm particles, Waters, Ireland) was used. The mobile phase consisted of 10 mM ammonium-formate in water (eluent A) and 10 mM ammonium-formate in methanol (eluent B), both pH = 4.0, adjusted with formic acid. The binary gradient program was as follows: 90% B (0 – 10 min), 90-50% B (10-15 min), 50% B (15-25 min), 50-40% B (25-30 min), 40% B (30-40 min), 40-10% B (40-50 min), 10% B (50-60 min), 10-90% B (60-75 min). The flow-rate was adjusted to 0.5 mL/min and the column was operated at 25°C. The injection volume of standard solutions and propolis samples was 5 μL. The detection of compounds was performed at 270 nm and UV spectra were recorded within a range of 200–400 nm, while quantification was performed at the λ_{max} for each standard. Identification of compounds in propolis samples was achieved by comparing their retention time values and UV spectra with those of investigated standards. Calibration curves of peak area versus analyte concentration were plotted from varying concentrations of commercially available standards - caffeic acid, *p*-coumaric acid, ferulic acid, isoferulic acid, quercetin, homoeridictyol, genistein, kaempferol, apigenin, rhamnetin, sakuranetin, isosakuranetin, chrysin, acacetin, galangin, pinostrobin, naringenin, tamarixetin, pino-cembrin, chrysin dimethylether, apigenin trimethylether (appropriate dilutions of standards were prepared from stock solutions, concentration 0.1 mg/mL). Concentrations of the phenolic acids and flavonoids present in propolis extracts were calculated from integrated areas of the sample and the corresponding standards.

3.3. Spectrophotometric and thin-layer chromatographic (TLC) assays

3.3.1. Standard solution and sample preparation

Standard solution was prepared by dissolving caffeic acid, galangin and pinocembrin (1 mg of each) in 96% ethanol (3 mL). From the existing stock solution, dilutions of 0.001, 0.005, 0.010, 0.025, 0.050 and 0.125 mg/mL were prepared for the DPPH˙ assay, while 10 times more concentrated solutions were used for the ABTS˙+ assay. Extraction of crude propolis samples was performed according to the procedure published by Cvek *et al.* [19]. Briefly, raw propolis (1 g) was extracted with 80% ethanol (10 mL). The extraction mixture was stirred for 1 hour at the room temperature (21±2 °C), using a Labortechnik RCT basic (IKA, Germany) magnetic stirrer with heating control. Obtained mixture was then centrifuged at 1610 g for 10 min, the supernatant was quantitatively filtered (ready-to-use filter units FP 30/0,8 CA, Schleicher & Schuell, Dassel, Germany). Obtained propolis extracts were diluted 250, 500, 750, 1000 and 2000-fold for both spectrophotometric assays, while additional dilutions of 50 and 100 were used only for the ABTS˙+ assay. For TLC analysis eight

different concentrations of standard solution (in the 10 µg/mL to 45 µg/mL range) were prepared, as well as dilutions of propolis extracts (from 800 up to 3400).

3.3.2. Spectrophotometric assays

DPPH$^{\cdot}$ is a stable violet-colored free radical that exhibits a high absorption value at 518 nm. As its odd electron becomes paired off in the presence of a free radical scavenger, the absorption diminishes, and the observed degree of decolorization can be taken as a measure of the reducing capacity/antioxidative activity of tested plant extract compounds. DPPH$^{\cdot}$ scavenging activity was measured essentially as described by Cotelle *et al*. [7]. DPPH$^{\cdot}$ was dissolved in 96% ethanol to obtain a 0.3 mM solution. Standard solutions, propolis extracts or ethanol itself as a control (2.5 mL) were mixed with DPPH$^{\cdot}$ solution (0.5 mL) and the decrease in absorption at 518 nm was recorded after 10, 20, 30, 60 and 120 seconds.

In our work we used an improved ABTS$^{\cdot+}$ radical cation decolorization assay, according to the published paper of Re *et al*. [10]. The improved technique for the generation of ABTS$^{\cdot+}$ involves the direct production of the blue/green ABTS$^{\cdot+}$ chromophore through the reaction between ABTS and potassium persulfate. Addition of the antioxidants to the pre-formed radical cation reduces it to ABTS, to an extent and on a time-scale depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. Thus, the decolorization of the ABTS$^{\cdot+}$ radical cation (followed as a decrease in absorbance at 730 nm) is determined as a function of antioxidant concentration and time. For the purpose of the described analysis, ABTS was dissolved in water to obtain a 7 mM concentration. ABTS radical cation (ABTS$^{\cdot+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at the room temperature for 16 hours. After mixing standard solution or propolis extracts (30 µL) with ABTS radical cation solution (3 mL), we monitored the decrease of absorbance after 30, 40, 50, 60 and 120 seconds. All spectrophotometric measurements were performed in triplicates, using Carry 50 UV-VIS spectrophotometer (Varian, UK), and the average values were taken into further calculations.

3.3.3. Thin-layer chromatography

For TLC analyses we used 5x20 cm glass-backed plates coated with 0.2 mm layer of silicagel 60 F$_{254}$ (Merck, Darmstadt, Germany). Standard solutions and diluted extracts (2 µL) were applied in the form of 20 mm bands to the plate using a Linomat 5 system (CAMAG, Switzerland). Plates were than sprayed with 0.3 mM DPPH$^{\cdot}$ and 0.7 mM ABTS$^{\cdot+}$ solutions, prepared in the same manner as for the spectrophotometric measurements. Plates were dried for 30 seconds at the room temperature. Considering the evaporation of the solvent and possible oxidation processes that can occur when the plate is exposed to air, our preliminary results suggested (data not shown) that it was the best to perform analyses after 30 seconds. The greatest difference between background and sample band color was noticed after that time period. The amount of transformed purple DPPH$^{\cdot}$ radical into the yellow reduced form was evaluated as the fluorescence intensity of bright (yellow) spot on the purple background at 518 nm. Procedure for evaluation of ABTS$^{\cdot+}$ radical cation amount was similar; we measured the intensity of fluorescence at 730 nm of bright (light-yellow) spot on the green

background. All measurements were performed in triplicates, using scanning densitometry (Scanner 3 system, CAMAG, Switzerland), and the further calculations were performed by using the average values.

3.4. Statistical Analysis

Statistical analysis of all obtained results was performed using STATISTICA v7.0 software (StatSoft, USA). To establish the correlation between the concentrations of flavonoids, phenolic acids and antioxidative efficiency (*AOE*) of used propolis samples, determined by using different assays, multiple linear regression was applied. The following statistical parameters were observed: *n* (the number of observation), *r* (the regression coefficient), *s* (the standard error of estimation) and *F* (the calculated value of *F*-test).

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