Analytical study of the determination of flavonoids in Black Sea algae

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Abstract This paper reports the results of flavonoids determination expressed in rutin from marine algae. For this purpose, rutin and rutin - $AlCl_3$ complexes were spectrophotometrically studied in three domains of ligand concentrations: ligand C_{rut}=1.34-6.7mg/L, 4-20mg/L and 2-32mg/L.
The precision, accuracy, specificity, and the quantification limit of the method were verified. The calibration curve traced for the concentrations domain 2-32mg/L rutin - $AlCl_3$ was used to determine the flavonoid content expressed in rutin of Black Sea algae.
The flavonoid content of the samples expressed in mg rutin/100g dry alga differs significantly: *Ulva lactuca*: 0.65mg/100g, *Cystoseira barbata* L: 2.35mg/100g, *Cladophora vagabunda L:* 6.14mg/100g, and *Enteromorpha intestilalis*: almost 23mg/100g dry alga.
The results of this study join other determinations applied to the same samples for the characterization of the active principles in marine algae, in order to use them for therapeutic purposes.

Keywords: rutin, marine algae, complexation, flavonoids

1. Introduction

Economically, marine algae can be used for various purposes, such as: in agriculture, in pharmaceutical and food industry, as they are rich in organic substances, sugars and active principles.

Flavones are mainly found in cereals and herbs. In the past years, scientific and public interest in flavones has grown enormously [1] due to their putative beneficial effects against atherosclerosis, osteoporosis, diabetes mellitus and certain cancers [2]. The intake of flavones in the form of dietary supplements and plant extracts has increased steadily.

Flavonoids form internal complexes, chelate type, with Al^{3+} according to the reaction (**Fig.1**). These compounds are extractible in hydrophilic solvents [3].

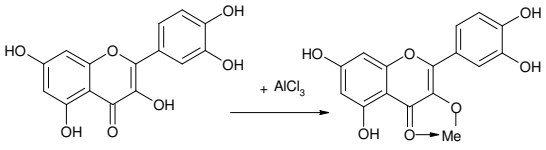

Fig.1 The chemical reaction flavone determination is based on

The intensity of the yellow color of the kelate formed by the flavonoids when treated with $AlCl_3$ in acetate buffer was spectrophotometrically determined [4]. The calibration curve is used to determine the whole flavonoid content expressed in rutin. Marine algae extracts treated with the same reagents were analysed under the same spectrophotometric conditions in order to determine the total flavonoid content expressed in rutin.

2. Experimental

2.1. Standard and apparatus

The rutin from Merck was used for the preparation of the standard solutions (Rutin Quercitin-3-L-rhamnoside, (Quercitin (3,3',4',5,7-pentahydroxyflavone-3-L-rhamnopyranoside, $C_{27}H_{30}O_{16}$ $3H_2O$; M = 664.6).

The determinations were performed with SPECTROMETER CINTRA 10e UV-VIS spectrophotometer with the following specifications: Wavelength Range 190-1.200nm; Monochromator Czerny-Turner mounting with holographic grating and automatic lamp peaking; Spectral Band pass 1.5nm; Scanning Speed 15.000nm//minute; Detector Silicon Photodiode.

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2.2 Spectrophotometric study of rutin standard solutions.

The rutin standard solutions with the same analytic concentration (C_{rutin} =13.4mg/L) diluted in buffers with different pH values were spectrophotometrically analyzed. The overlaid spectra illustrate the change of the spectral profile due to the change in the chromophore structure. The spectra with pH 4-5 have absorbance maximums around 350nm. This pH was considered optimum for complex analysis to better differentiate between the spectral profile of the ligand (rutin, **Fig. 2**) and the rutin/Al^{3+} complex (**Fig. 3**).

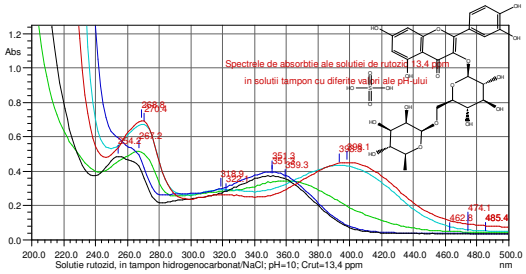

Fig. 2. The absorption spectra of rutin standard solutions in buffers with pH values between 1 and 10 (C_{rutin}=13.4mg/L), within the range 250-500nm

2.3 Spectrophotometric study of Al^{3+}/rutin

The rutin/Al^{3+} complexes in acetate buffers were also spectrophotometrically studied according to the standard method [4]. In order to find the optimum concentrations range, the complex was studied on three domains of ligand concentrations: 1.34-6.7mg/L, 4-20mg/L and 4-32mg/L respectively.

The absorbance spectra of rutin/ Al^{3+} complex in acetate buffers (C_{rutin}=1.34-6.7mg/L), in 250-500nm interval do not intersect, which proves the existence of a single chemical species: the complex.

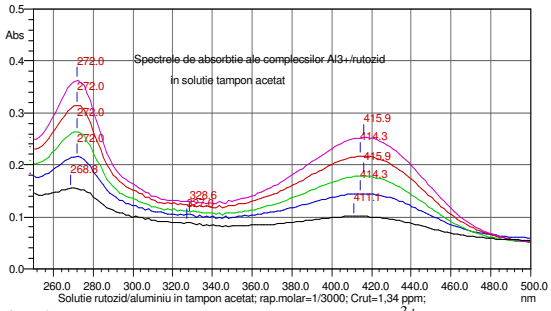

Fig. 3 The absorption spectra of rutin/Al^{3+} standard solutions in acetate buffer (C_{rutin}=1.34-6.7mg/L), in the interval 250-500nm

The overlaid spectra of the standard solution of rutin and rutin/Al^{3+} complex with the same rutin content (C_{rutin}=6.7mg/L), in acetate buffer, in the interval 250-500nm, illustrate the movement of the absorbance maximum of the complex towards longer wavelength (bathochromic schift) and the intensification of the absorbance maximum (hyperchromic effect), which proofs the kelate formation (**Fig. 4**).

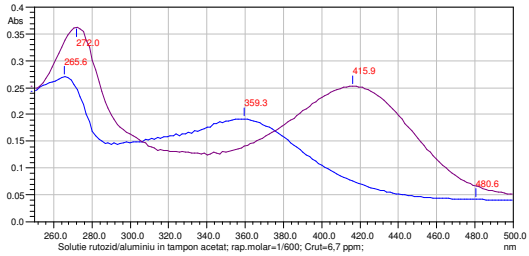

Fig. 4. Overlaid spectra of standard solution of rutin (C_{rutin} =6.7mg/L) and rutin/Al^{3+} complex, with the same ligand content, in acetate buffer solution, within the range 250-500 nm

The calibration curves traced for standard solutions of rutin/Al^{3+} complex with C_{rutin}=1.34-6.7mg/L concentrations at two wavelengths of 415 nm (**Fig.5**) and 420 nm indicate that the reproducibility coefficient has better values at 415 nm. Thus, in order to determine the flavonoid content expressed in rutin in lower concentrations (below 10 mg/L), it is recommended that the equation of the calibration line be at 415 nm (**Table 1**).

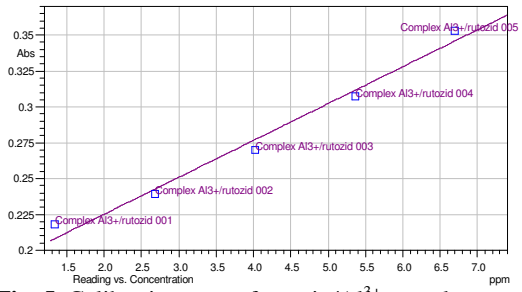

Fig. 5. Calibration curve for rutin/Al^{3+} complexes at 415 nm (C_{rutin}=1.34-6.7mg/L)

The equation of calibration curve at 415nm:

$$C_{rut}=-6.769+38.87\times A_{415nm}$$

Correlation coefficient: r =0.989980

The equation of calibration curve at 420nm:

$$C_{rut}=-3.858+41.10\times A_{420nm}$$

Correlation coefficient: r = 0.976351

Standard solutions of rutin /Al^{3+} were also prepared on two domains of concentrations: 4-20 mg/L and 2-32 mg/L (**Table 2**) in order to use the best calibration curve adequate to sample concentration.

Table 1. Results of the quantitative analysis of standard solutions of rutin/ Al^{3+}complexes at 415 nm and 420 nm (C_{rutin}=1.34-6.7mg/L)

Nr.	$C_{rut.}$ mg/L	Mol ratio rutozid/Al^{3+}	A 415nm	RSD%	A 420nm	RSD%
1	1.34	1/3000	0.21832	0.03576	0.13855	0.00860
2	2.68	1/2000	0.23923	0.06568	0.16031	0.07317
3	4.02	1/1000	0.26963	0.01297	0.17393	0.03244
4	5.36	1/750	0.30745	0.04669	0.22299	0.04148
5	6.70	1/600	0.35314	0.03512	0.26261	0.11077

Table. 2. Preparation of standard solutions of rutin/ Al^{3+} complexes with 2-32 mg/L rutin concentrations

Etalon	$V_{S,rut}$ 100mg/mL mL	$V_{S,acetate}$ 10% mL	$V_{S,AlCl3}$ 2,5% mL	$V_{ethanol}$ mL	V_f mL	$C_{rut.}$ mg/L	A 430 nm
1	0.2	2	1	6.8	10	2	0.064
2	0.5	2	1	6.5	10	5	0.150
3	0.8	2	1	6.2	10	8	0.242
4	1.1	2	1	5.9	10	11	0.341
5	1.4	2	1	5.6	10	14	0.420
6	1.7	2	1	5.3	10	17	0.510
7	2.0	2	1	5.0	10	20	0.592
8	2.3	2	1	4.7	10	23	0.680
9	2.6	2	1	4.4	10	26	0.765
10	2.9	2	1	4.1	10	29	0.846
11	3.2	2	1	3.8	10	32	0.931

The absorbance spectra of the standard solutions of rutin/ $AlCl_3$ with 2-32 mg/L in 400-600 nm interval (blank: 3 mL water and ethanol at 10 mL) was also studied. After 30 min, the solutions absorbance is determined at λ = 430 nm, in 1 cm cuvette. The absorbance spectra have maximum 430 nm, the absorbance has optimal values (**Fig. 6**) and the calibration curve is very good (**Fig.7**).

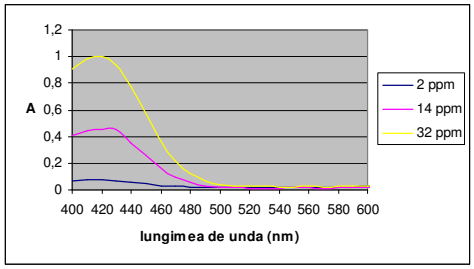

Fig. 6. Absorbance spectra of standard solutions of rutin/Al^{3+} complexes in acetate buffer (C_{rutin}=2-32mg/L) in the range 400-600 nm

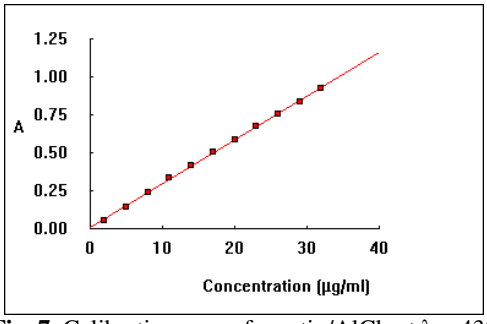

Fig. 7. Calibration curve for rutin/$AlCl_3$ at λ = 430 nm. 2-32 mg/L rutin concentrations.

The equation of the calibration curve is

$$C=-0.428+34.602\times A_{430nm}$$

Correlation coefficient r = 0.9998;
Coefficient of determination r^2 = 0.9996

To verify the specificity of the method of determination of flavonoids through complexing with $AlCl_3$, it was tested the complexation of other polyphenolic compounds. Tannin and caffeic acid do not form chelate with ion Al^{3+}, either in acidic buffer medium (pH = 5), or basic buffer medium (pH = 10), the analyzed solution remains colorless in the presence of $AlCl_3$. The anthocyanic extract forms violet kelate with ion Al^{3+} in basic alkaline solution (pH = 10), with the absorbance maximum at λ = 550 nm.

The tests proved that the presence of tannin in the buffer solutions of rutin does not change the results of the spectrophotometric determination of flavonoids through complexing with $AlCl_3$.

In order to verify **reproducibility of results**, the calibration curves were made with more concentrations of reference substance for three days consecutively, to determine the standard deviation (s) and variance (s^2) between different time intervals and during a certain time interval [5].

Simultaneously, during the three days of testing, we prepared samples of quality control (QC) under the same circumstances, but independently from standard curves. The concentrations of QC, corrected with "r' value for the calibration curve in the respective day, were compared to the values of the calculated concentrations [5].

In order to establish the **precision of the method**, by comparing the quantity of determined substance to the quantity of calculated substance, we performed a statistic analysis using the program ORIGIN 7 [5].

To verify the linearity of the method, we used the method of the smallest squares, on the 2-32 µg/mL domain for rutin. The correlation was considered linear at a value of the determination coefficient of r^2>0.99 [6].

On the basis of five measurements on five different samples at five levels of concentration for each reference substance (2, 5, 8, 11, 14 µg/mL) for rutin, we determined the accuracy, expressed as a relative percent deviation of the determined concentration, as opposed to the calculated concentration and the precision of method, expressed through the relative percent standard deviation or the coefficient of variation CV%, according to the calculation relations given below:

$$\text{Precision}\% = \frac{\text{standard deviation of the average}}{\text{average of determined concentration}} \times 100$$

$$Accuracy\% = \frac{C_{det.} - C_{calc.}}{C_{calc.}} \times 100$$

Accuracy and precision remained between ±20% limits at the lower calibration limit and ±15% at the other concentrations. They did not present a continuous rising or falling tendency with concentration [5].

The sensitivity limit was considered as the lowest concentration on the calibration line with accuracy and precision between ±20% limits [5].

2.4. Analysis of algae samples

The harvesting of algae was made by the Romanian Institute for Marine Research and

Development from an unpolluted area in the Black Sea. Algae were dried at room temperature in darkness and fine grounded.

Flavonoid extraction. 2 × 50 mL ethanol 50% was added to 5g algae powder and the mixture was brought to the boiling point, on the water bath for 30 min. The hot solutions were filtered; after cooling, they were put into a graduated flask of 100mL, washing the residue with the same solvent.

Preparation of working solutions. The samples with the obtained solution were prepared, taking 0.2mL, 0.5mL, 0.8mL, 1.1mL, 1.4mL, and 1.7mL of extract, 2mL of sodium acetate solution 100g/L, 1mL aluminium chloride 25g/L, and water, into a graduated flask of 10mL [7].

Simultaneously, the blank samples were prepared with corresponding concentrations of analyzed solution and 10mL water. After 30 min., the sample absorbance at 430nm was determined, and the obtained data were interpolated in rutin standard curve to obtain their concentration (**Table 3** and **Fig. 8**).

To calculate the quantity of total flavones (expressed in g rutoside at 100g vegetal product), the dilution factor was quantified, concentration calculated from the standard curve and sample mass:

$$C = \left[\frac{V_{s,flask}(mL) \times 10^{-3}}{m_{sample}(g)} \times F \times C_{det.} \left(\frac{mg}{mL} \right) \right] \times 100 \quad \frac{g\ flavone}{100g\ sample}$$

where: V_s – volume of solution prepared from the vegetal product, in volumetric flask

$m_{sample.}$ – mass of vegetal product

F – dilution factor

$C_{det.}$ – concentration determined from standard curve

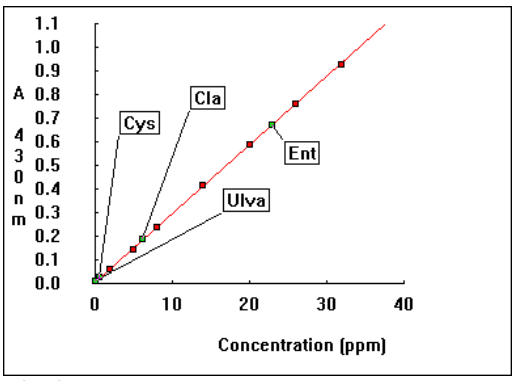

Fig. 8. Results of the determination of flavones from algae.

3. Results and Discussions

The calibration curve for rutin at wavelengths of 415nm, 420nm, and 430nm underlined correlation coefficients (r) with closed values; the highest is at λ=430nm, r=0.999799.

To calculate the content of total flavonoids, a standard curve was used, traced with known quantities of reference substance, rutin (Merck, M=664.5) 100mg/L in ethanol. The standard curve for rutin on the domain of concentrations 2-32mg/L and 1-7mg/L was traced, by measuring the absorbance of solutions at 30 min after preparation at λ=430nm, in 1cm cuvette.

Table 3. Results of determination of flavones from extracts from the algae *Ulva lactuca* L, *Cystoseira barbata* L, *Cladophora vagabunda L* and *Enteromorpha intestinalis* L

Analysed vegetal product	Mass p.V. g	$V_{sol.A}$ mL	V_f mL	A 430nm	Concentration from calibration curve ($C_{det.}$) mg/L	% flavones mg/100g
Cystoseira barbata L. (Cys)	1	3.33	10	0.028	0.235	2.352
Ulva lactuca L. (Ulva)	1	3.33	10	0.013	0.066	0.654
Cladophora vagabunda L. (Cla)	5	2	10	0.190	3.073	6.146
Enteromorpha intestinalis L. (Ent)	5	2	10	0.675	11.469	22.928

The standard curve for rutin on the domain of concentrations 2-32mg/L and 1-7mg/L was traced, by measuring the absorbance of solutions at 30 min after preparation at λ=430nm, in 1cm cuvette.

From the point of view of the stability of solutions prepared when calibration curves were established, it was observed that in 24h the rutin solutions precipitated (the determination of absorbance is not possible anymore). With the help of the absorbance values determined during the three days of testing, we traced standard curves and established the equations of the calibration curve and the correlation coefficients for each curve. The coefficients of determination are higher than 0.997 [5].

Day 1. $A_{430nm} = 0.0004 + 0.0329 \times C_{rut.}$
$r^2 = 0.9991$
Day 2. $A_{430nm} = 0.0178 + 0.0332 \times C_{rut.}$
$r^2 = 0.9973$
Day 3. $A_{430nm} = 0.041 + 0.033 \times C_{rut.}$
$r^2 = 0.9984$

The methods have a linear response on the chosen domains, with an average coefficient of determination $r^2 > 0.998$. The averages of the determined concentrations are in the interval of trust 95% [8].

The accuracy and precision of the determinations performed on rutin are within the limits admitted in such determinations (±20% at limit of quantification, ±15% respectively at other concentrations) [9]. The limit of quantification was established at 2µg/mL for rutin, with accuracy and precision between the admitted limits of ±20% [10].

4. Conclusions

The spectrophotometric methods of the determination of flavonoids are specific, sensitive, precise, reproducible, which makes their use in the evaluation of flavones content of samples and the standardization of tinctures and soft extracts obtained from them sure.

Tests show that the optimal wavelength for 1-7 mg flavones /L in samples is 420nm, while for concentrations in the range 2-32 mg flavones/L, is.430nm.

The flavonoid content of the samples expressed in mg rutin/100g dry alga is very different: *Ulva lactuca*: 0.65mg/100g, *Cystoseira barbata* L: 2.35mg/100g, *Cladophora vagabunda L:* 6.146mg/100g, and *Enteromorpha intestinalis*: almost 23mg/100g dry alga.

5. Acknowledgements

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6. References

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[1]. R. Cermak and S. Wolffram Curr. Drug Metab. **7** (7) 729–44. (2006)
[2]. R Cermak, Expert Opin Drug Metab Toxicol **4** (1) 17–35, (2008)
[3]. V. Istudor, *Farmacognozie. Fitochimie. Fitoterapie*, vol. I, Editura Medicală, Bucureşti, (in Romanian) 1998, 76-90, 112-118, 145-204.
[4]. *** Farmacopeea Română Ediția a X-a, Editura Medicală, Bucureşti, 1993, 921-922, 1124-1131.
[5]. L. Bucur, C. Sava, L. Petcu and V. Istudor, Revista Medico-Chirurgicală a Societății de Medici şi Naturalişti din Iaşi, (I Romanian) **II(2)**, Suplimentul nr.1, 74-78 (2008).
[6]. N.P.Bekker and A.I.Glushenkova, Chemistry of Natural Compounds **37** (2) 97-116. (2001)
[7]. L. Bucur, M. Arcuş, A. Popescu, S. Vameşu and V. Istudor, Ovidius University Annals of Medical Science-Pharmacy, **1** (2), 29-32 (2003).
[8]. L. Popa, *Elemente de metodologia cercetării ştiințifice în domeniul farmaceutic*, ediția a II-a revizuită şi adăugită, (in Romanian) Editura Printech, Bucureşti, 2005, 187-192.
[9]. L.C. Petcu *Biostatistică. Teorie şi aplicații în domeniul medical,* Ovidius University Press, Constanța, 2005.
[10]. L.Vlase, S. Leucuța and S. Imre, Rev. Chim., **58** (1), 3-7 (2007).