DETERMINATION OF ANTHOCYANINS IN SOME VEGETABLES AND FRUITS BY DERIVATIVE SPECTROPHOTOMETRIC METHOD

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ABSTRACT

Anthocyanins (E 163) have been determined in red cabbage, blackberry, morello cherry, grape and sumac fruit by first order derivative spectrophotometry without using any separation or background correction techniques and reagents. The method is based on the measurement of the distances between two extremum values (peak-to-peak amplitudes) in the first order derivative spectra of the extracts. In the developed method, perchloric acid (5% v/v) was found to be the most suitable extraction solution. The extraction time was determined as 10 minutes in fresh fruits and vegetables and in dried ones 20 minutes. In the first order derivative spectrum, the maximum at 490.5 nm and the minimum at 550.2 nm were used for the quantitative determination of anthocyanins in red cabbage, sumac fruit, blackberry, morello and grape. A calibration curve was constructed for the 100-500 μg mL^{-1} concentration range. The calibration equation and regression coefficient obtained from the first derivative spectra is $^1D_{490.5\text{-}550.2}$= 0.0543C + 0.926, r=0.9999.

Key words: Anthocyanins determination, derivative spectrophotometry, fruits, and vegetables.

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INTRODUCTION

Anthocyanins (Ac) are water-soluble vacuolar flavonoid pigments that appear in a range from red to blue, according to the pH of the vacuole water; they are therefore responsible for colors (purple, blue and red) in many plants. Approximately 550 different anthocyanins are known. The difference in chemical structure that occurs in response to changes in pH is the reason why anthocyanins are often used as pH indicators, as they change from red in acids to blue in bases.

The anthocyanins are widely distributed in nature, occurring in higher plants. They are found in all parts of the plant but are most obvious in fruits and flowers /1/. In the European Economic Community (EEC) countries, anthocyanin extracts from food sources are generally allowed (for specific information this class of colorants is listed under EEC E163). In addition to extracts, the concentrated juice of red fruits such as cranberries, raspberries, elderberries, etc., can also be used in food products that are compatible with the acidity and flavor of the fruit juice concentrate involved. In the United States and for the most EEC countries, fruit juice and concentrates can be used without restriction.

Anthocyanidin	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Aurantinidin	-H	-OH	-H	-OH	-OH	-OH	-OH
Cyanidin	-OH	-OH	-H	-OH	-OH	-H	-OH
Delphinidin	-OH	-OH	-OH	-OH	-OH	-H	-OH
Europinidin	$-OCH_3$	-OH	-OH	-OH	$-OCH_3$	-H	-OH
Luteolinidin	-OH	-OH	-H	-H	-OH	-H	-OH
Pelargonidin	-H	-OH	-H	-OH	-OH	-H	-OH
Malvidin	$-OCH_3$	-OH	$-OCH_3$	-OH	-OH	-H	-OH
Peonidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	-OH
Petunidin	-OH	-OH	$-OCH_3$	-OH	-OH	-H	-OH
Rosinidin	$-OCH_3$	-OH	-H	-OH	-OH	-H	$-OCH_3$

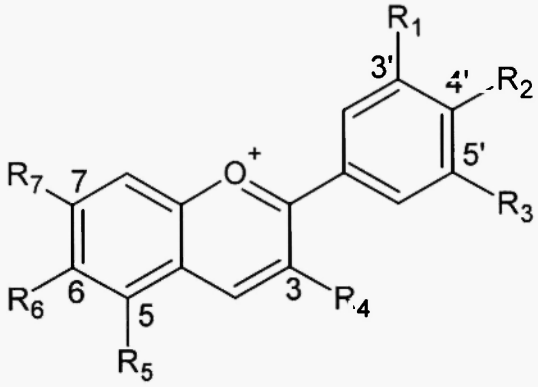

Fig 1: Structural formulas of the anthocyanins [2]

Anthocyanins are becoming increasingly important not only as food colorants, but also as antioxidants. Anthocyanins are reported to have some therapeutic benefits including vasoprotective and antiinflammatory properties /3/, anticancer and chemoprotective properties /4/, as well as antineoplastic properties /5/. Anthocyanins are therefore, considered to contribute significantly to the beneficial effects of consuming fruits and vegetables /6/.

The recent banning of Red No.2 in the United States along with the questionable status of red No. 40 has led to the increased use of natural red pigments as coloring agents. The anthocyanins are one such class of natural red pigments that have found use as a suitable alternative for synthetic colorants in many applications /1,7/.

The demand for anthocyanin containing colorants is increasing. Fruit juice concentrates have become an important ingredient in the manufacture of many foods and beverages. Production of fruit juice concentrates has become highly competitive in both domestic and foreign markets. Analytical methodology able to identify the anthocyanins and to determine their source are needed for quality control and for determination of the authenticity of fruit juice concentrates and color extracts as well as for regulatory activities /8/. The concentration of Ac in vegetables and fruits is a maturity index, and the determination of this constituent in such samples is of special interest in quality control.

The need for a fast and selective method is obvious, especially when routine determinations are required. Many analytical methods have been developed for the determination of Ac and the quality control of products

containing Ac. These include spectrophotometric /9-12/, high-performance liquid chromatography (HPLC) /13-16/, capillary zone electrophoresis (CZE) /17/, fourier transform infrared spectroscopy /18/, liquid chromatograph-diod array detection mass spectrometry (LC-DAD-ESI/MS) /19,20/, HPLC/photodiode array detection /7/, as well as combination of other techniques /21,22/.

Spectrophotometry is a fast and simple method for Ac determination, but it cannot be used in samples with complex matrices because of background absorption in the UV-vis region. Various background correction techniques have been proposed such as thermal decomposition, direct ultraviolet irradiation, alkaline treatment and an enzymatic method /23,24/. Some visible spectrophotometric methods often require tedious pre-separation techniques to remove possible interferences from colored materials. Derivative spectrophotometry is a useful technique for eliminating the effect of baseline shifts and baseline tilts /25-28/.

The purpose of this work is to develop a direct and simple spectrophotometric method for the determination of Ac in fruits and vegetables without pre-separation or background correction procedures. Among fruits and vegetables, red cabbage, blackberry, morello cherry, grape and sumac were chosen owing to their high Ac content.

EXPERIMENTAL

Apparatus

A Philips PU 8700 UV-Vis spectrophotometer was used with a cell having a path length of 1cm and a volume of 3 mL glass cells (Hellma, 100-QS). For the spectrophotometric measurements, suitable settings were chosen: 2 nm slit width, 1000 nm min^{-1} scan speed, very high smoothing. A mechanical blender (Bosch 1210, 500W, 27000, 1/min), an analytical balance (Sartorius, analytic A 200 S), Whatman 541 filter paper and technical glass materials were used.

Reagents

All chemicals and solvents used were analytical reagent grade and purchased from E. Merck, Darmstadt. Redistilled water was used throughout the work.

Acetic acid (96%, 1.06 kg/L), m-phosphoric acid (80%, 1.15 kg/L), o-phosphoric acid (80-85%, 1.71 kg/L), perchloric acid (70-72%, 1.67 kg/L), citric acid (99%, 1.08 kg/L), formic acid (89-91%, 1.19 kg/L) and hydrochloric acid (37%, 1.19 kg/L) were used.

Analytical Procedures

Preparation of Stock and Standard Solutions

Stock solution of anthocyanins (1 mg L^{-1}) was prepared with (5% v/v) perchloric acid solution to determine the experimental conditions and constructed of standard curves.

This solution was diluted to obtain standard solutions (100-500 μg ml^{-1}) for the preparation of calibration curves. These solutions were freshly prepared every day.

Extraction of Anthocyanins

Effect of the different acids on the extraction of anthocyanins

An accurately weighted amount of 0.5 g of homogenized red cabbage sample was extracted with 50 mL perchloric acid (5% v/v) solution using a mechanical stirrer for about 30 min. The filtrate was transferred into 100 mL volumetric flask by using filter paper and diluted to an appropriate volume with the same solution. The same procedure was repeated with the same percentage of formic acid, hydrochloric acid, acetic acid, citric acid, m-phosphoric acid, o-phosphoric acid. Absorption spectra of sample solutions were recorded against the extraction solution.

Determination of the ratio of material / solvent

Homogenized red cabbage samples were weighted amounts of 0.5; 1.0; 1.5; 2.0; 2.5 and 5.0 grams respectively and extracted with 50 mL of perchloric acid (5% v/v) by using a mechanical stirrer for about 30 min. The extracts were filtered through into 100 mL volumetric flasks separately and diluted to an appropriate volume with extraction solution. The same procedure was repeated for blackberry, morello cherry, grape and sumac

separately. Absorption spectra of filtrates were recorded against the extraction solution.

Effect of acid percentage (%) on the extraction of anthociyanins

In the literature, many different acid percentages have been used for extracting Ac from plant materials. In the investigation of the literature, it was seen that most workers employ (0.1%; 0.6%; 4.0%; 5.0%; 10.0%; 15.0%) acid percentage for extracting Ac /29/.

To determine percentage of perchloric acid, different percentage solutions (0.2%, 0.5%, 1.0%, 5.0%, 10.0%, 15.0% v/v) were prepared in our research. An accurately weighted amount of 0.5 g of homogenized red cabbage sample was extracted with 50 mL (0.2 % v/v) perchloric acid solution using a mechanical stirrer for about 30 min. The filtrate was transferred into 100 mL volumetric flask and diluted to an appropriate volume with perchloric acid (2%, v/v) solution. Absorption curve of filtrate was recorded. The same procedure was repeated for (0.5%, 1.0%, 5.0%, 10.0%, 15.0% v/v) perchloric acid solutions.

Effect of extraction time on the extraction of anthociyanins

Research reports showed that the extraction time depends on different materials and agitators, that is, it shows differences .To determine the extraction time, homogenized red cabbage samples were weighted amount of 0.5 gram separately and stirred with 50 mL of perchloric acid (5% v/v) solution for different contact time (2.0, 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 min) then the extracts were filtered into 100 mL volumetric flasks and diluted to an appropriate volume with extraction solution. Absorption spectra of filtrates and the absorbance values of λ_{max} = 520 nm were recorded.

Calibration Graphs

Absorption and the first order derivative absorption spectra of anthocyanins standard solutions (100-500 μg ml^{-1}) were recorded between 450-600 nm. In these spectra, the absorbance values of λ_{max} = 520nm and the peak-to-peak amplitudes of 490.5 nm and 550.2 nm were used for the establishment of the calibration graphs for fruit and vegetables samples. The regression equation of the calibration graphs were calculated by the method of least squares.

Preparation of Sample Solutions

Samples were purchased from the local markets for analysis. Sumac was bought in powder form. Red cabbage (0.5 g), blackberry (0.5 g), morello cherry (0.5 g), grape (5 g) and sumac (4 g) samples were chopped and then homogenized with 50 mL extraction solution (5% v/v) perchloric acid-water) using a mechanical stirrer (with a glass rod) for about 10 min. After homogenization of the samples, the extracts were transferred into 100 mL volumetric flasks by using Whatman 541 filter papers and diluted to provide the necessary working concentration (100-500 μg ml^{-1}). The filtrates were used to determine amounts of anthocyanines by absorption and derivative spectrophotometry.

Sample analysis

Absorption and the first order derivative spectra of sample solutions were recorded against the extraction solution. The absorbance values at λ_{max} = 520nm and peak to peak amplitudes of 490.5-550.2 nm were obtained from these spectra.

The concentration of anthocyanins in the sample solutions was deduced by means of the regression equations of the related calibration graphs.

RESULTS AND DISCUSSION

A spectrophotometric method cannot be used directly for the determination of anthocyanins in fruits and vegetables owing to the matrix effect of UV-vis absorbing substances in sample matrix. This effect is clearly seen in Fig. 2, which shows the absorption spectra of the red cabbage, blackberry, morello cherry, grape and sumac extracts, together with the spectra of standard anthocyanins solutions prepared in the same solvent medium. To overcome this difficulty, some tedious background correction techniques have been used /23-28/.

In contrast, the derivatization of the absorption spectrum and measurement of the distance between two neighboring extremum values allow the elimination of matrix effects, because the valuable background absorptions overlapping the analyte peaks are smoother in derivative spectra. Figure 3 shows first derivative of the absorption spectra given in Fig. 2.

In the works published up to now, formic acid, hydrochloric acid, acetic acid, perchloric acid, citric acid, m-phosphoric acid, o-phosphoric acid have been used for extracting Ac from fruits and vegetables /30/. Perchloric acid was found to be the most suitable extraction solution among the various acids tested for the extracting Ac from fruits and vegetables (Table 1). The influence of percentage of perchloric acid in the range of 2% to 15% (v/v) on the extraction of Ac has been studied. It was determined to be the most suitable percentage 5.0%, (v/v) among the different percentages tested for the extraction Ac such as 0.2%, 0.5%, 1.0%, 5.0%, 10.0% and 15.0% (Table 2). The optimum extraction time was determined as 10 minutes for red cabbage, blackberry, morello cherry and sumac. However, 10 minutes was not enough for grape and the most suitable extraction time was found to be 20 minutes. These results can be seen from Fig. 4.

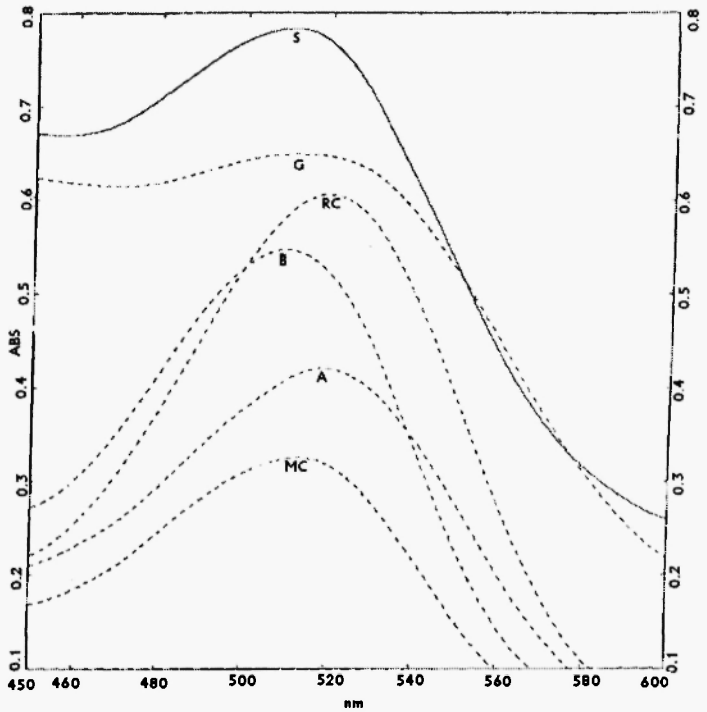

Fig 2: Absorption spectra of sumac (S), grape (G), red cabbage (RC), blackberry (B), anthocyanins (A) and morello cherry (MC) solutions.

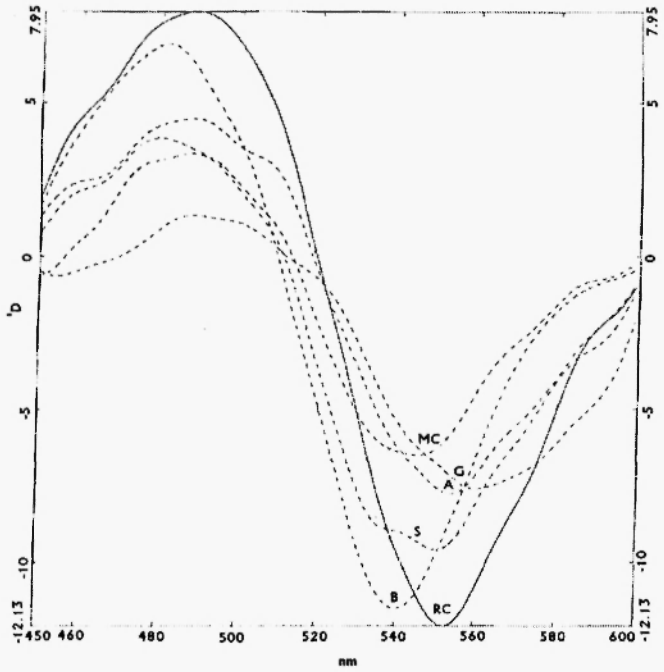

Fig 3: First order derivative spectra of sumac (S), grape (G), red cabbage (RC), blackberry (B), anthocyanine (A) and morello cherry (MC) solutions.

Table 1

Effect of acids on the extraction of anthocyanins

Acid	Anthocyanins λ_{max}	Red Cabbage λ_{max}
Formic acid (HCOOH)	522.4	526.1
Hydrochloric acid (HCl)	520.8	522.9
Acetic acid (CH_3COOH)	523.6	529.3
Perchloric acid ($HClO_4$)	519.0	520.8
Citric acid $HOC(COOH)(CH_2COOH)_2$	522.4	525. 6
m-Phosphoric acid $(HPO_3)_n$	520.1	524.0
o-Phosphoric acid (H_3PO_4)	520.2	522.9

Table 2
Effect of perchloric acid percentage (% ,v/v) on the extraction of anthocyanins

Perchloric Acid Percentage (% v/v)	λ_{max} (nm)	Absorbance Values (A)
0.2	524.1	0.832
0.5	523.1	0.843
1.0	521.6	0.917
5.0	520.0	0.935
10.0	519.2	0.956
15.0	524.0	0.960

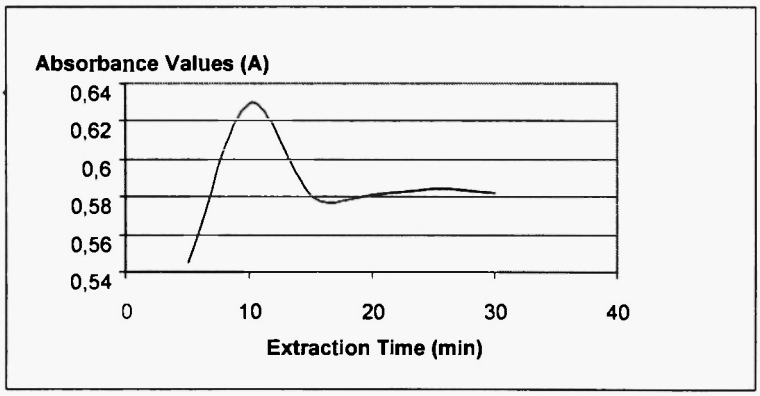

Fig. 4: Effect of extraction time

A linear relationship was obtained between Ac concentration (100-500 μg ml^{-1}) and absorbance values at λmax = 520nm. A linear relationship was also obtained between Ac concentration (100-500 μg ml^{-1}) and peak to peak amplitudes (^1D) of first order spectra at the wavelengths stated above.

Table 3 shows the calibration equations and regression coefficients obtained from the absorbance and first derivative spectra. The anthocyanins contents were determined from the absorbance values at λ_{max} 520 nm and the first derivative spectrum by measuring a maximum 490.5 nm and a minimum at 550.2 nm. The standard calibration curves are shown in Fig 5

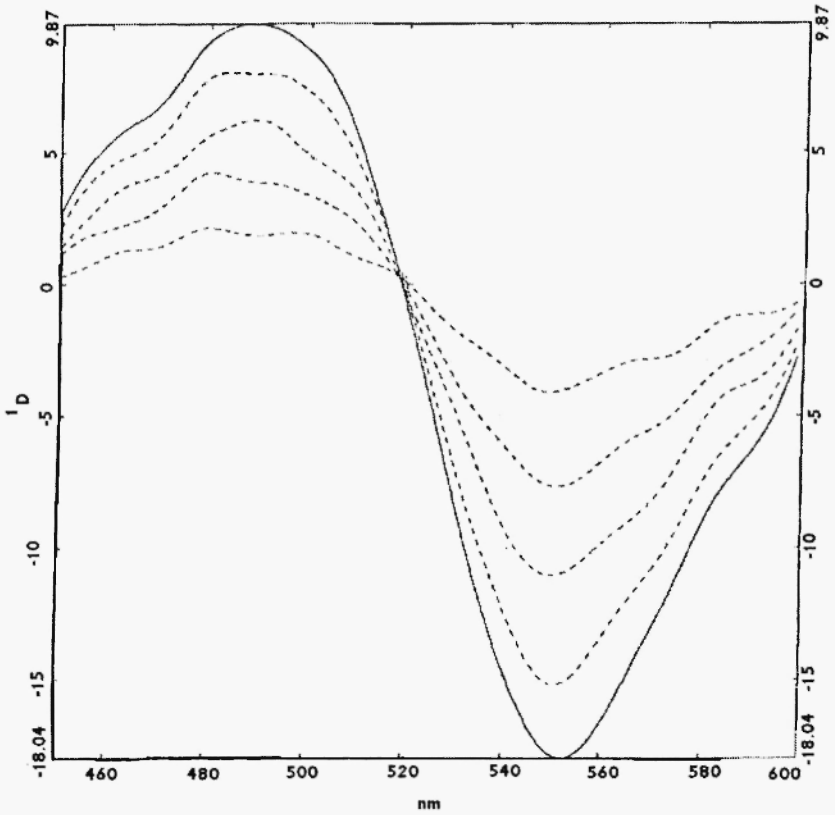

Fig 5: First order derivative spectra of Standard Anthocyanins solutions (100-500 μg mL^{-1})

Table 3
Calibration equations of anthocyanins

Equation	Regression Coefficient	Concentration Range
$A_{520} = 0.0021C_A - 0.0132$	r = 0.9999	100-500 μg mL^{-1}
$^1D_{490.5\text{-}550.2} = 0.0543\ C_A + 0.926$	r = 0.9999	100-500 μg mL^{-1}

C_A= Anthocyanins concentration (μg mL^{-1})

*Five separate workups were performed and the mean calculated.

The content of Ac in red cabbage, blackberry, morello, cherry, grape and sumac fruit were determined by the spectrophotometric and the proposed derivative spectrophotometric method. The results obtained by these methods were compared at the 95% confidence level with the aid of *F* test for precision (Table 4)

Mean values and standard deviations are also summarized in Table 4. As can be seen from Table 4, the calculated F values for red cabbage, blackberry, morello cherry (1.65, 1.81, 3.56, respectively) were less than the corresponding ones obtained from the standard table for the selected confidence level and population number. The F values for sumac and grape (7.90, 35.70, respectively) were higher because of matrix effects of the samples.

When the spectrophotometric method was applied to the determination of Ac for grape and sumac higher values were found due to the turbidity. This makes the derivative method particularly useful for quantitative determination in the presence of turbidity or when the background absorbance is higher not very well detailed. The first derivative method was applied to overcome this difficulty.

CONCLUSION

In conclusion, the derivative specrtrophotometric method is relatively easy, fast and cheap for determination of the Ac content of cabbage, blackberry and morello cherry, grape and sumac fruit. Because it does not require expensive solvents and reagents, it may be recommended for the rapid precise and sensitive quantification of Ac in these products. The major instrument required is a modern, microcomputerized Uv/vis spectrophotometer, which can be purchased at a reasonable price. This method also takes less time than the others. The method can be extended to Ac determination in various fruits and vegetables also grape and sumac fruit provided that the proper wavelengths for peak-to-peak measurements are accurately selected. For the selection, the first, second and third order derivative spectra of the sample extract and standard Ac solution in the same concentration and solvent medium are plotted on the same graph,and the region in which the two spectra completely overlapped was determined.

Table 4

Comparison of the results for Ac determination by the proposed method (^{1}D) and spectrophotometric method (A)

	Value obtained, mg Ac per 100 g								F_{theory}* = 5.05	
	Cabbage		Sumac		Black berry		Morello		Grape	
No	A	^{1}D	A	^{1}D	A	^{1}D	A	^{1}D	A	^{1}D
1	23.18	22 66	29.41	10.84	50.18	49.74	28.65	28.30	11.02	3.42
2	23.55	24.01	29.80	10.78	56.65	57.78	28.56	28.68	11.83	3.59
3	24.73	23.33	25.74	9.21	51.27	52 26	29.29	30.04	14.71	4.09
4	26.65	24.62	26.56	9.42	39.05	39.68	33.21	29.84	14.76	3.93
5	26.10	27.75	24.99	9.64	50.08	52.52	29.75	30.16	11.26	3.26
6	24.55	24.56	25.42	10.65	49.69	38.48	28.65	28.02	10.53	3.45
$\bar{x}$	24.79	24.49	26.99	10.09	49.49	48.41	29.69	29.17	12.35	3.62
S	1.37	1.76	2.10	0.75	5.73	7.70	1.79	0.95	1.89	0.32
%S	5.53	7.19	7.77	7.43	11.58	15.91	6.03	3.26	15.30	8.84
$\bar{x}$ ±	24.79 ±	24.49 ±	26.99 ±	10 09 ±	49.49 ±	48 41 ±	29.69 ±	29.17 ±	29.17 ±	29.17 ±
t.S/n^{½}	0.168	0.22	14.53	5.19	0.58	0.79	1.88	0.99	0.99	0.99
F_{calc}*	1.65		7.90		1.81		3.56		35.70	

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