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Isolation and Identification of a Pigment from Grape for Use as Acid-Base Indicator.

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ABSTRACT

This paper deals with the extraction of a pigment (Malvidin-3-fructoside) from the Iraqi Kurdistan grape (*Vitis vinifera L.*). The extracted pigment was purified using physical methods including different chromatographic techniques (TLC, PC, PTLC and HPLC). The pure extracted pigment was identified using different techniques such as infrared (IR), ultraviolet (UV), ^{1}H-NMR, ^{13}C-NMR and CHN analysis.

The isolated pigment from grape was suggested to be very useful indicator for acid-base titration due to good precision and accuracy compared with commercial indicators such as methyl orange, methyl red and phenolphthalein. This pigment was also used in the preparation of indicator paper.

عزل وتشخيص صبغة جديدة مستخلصة من عنب واستخدامه كدليل في تسحيحات الحامض –القاعدة

الملخص

يهتم هذا البحث باستخلاص صبغة المالفدين-3-فركتوسايد من العنب الموجود في كوردستان العراق. الصبغة المستخلصة تم تنقيتها باستخدام طرائق فيزيائية والتي تتـضمن طرائـق كروماتوغرافيـة مثـل كروماتوغرافيا الطبقة الرقيقـة وكروماتوغرافيـا الورقـة وكروماتوغرافيـا الطبقـة الرقيقـة الكميـة وكروماتوغرافيا السائل ذي الأداء العالي. أيضا تم تشخيص الصبغة باستخدام طيف الأشعة تحت الحمراء (I.R.) وطيف الأشعة فوق البنفسجية (U.V.). وطيف الرنين النووي المغناطيسي للبروتـون والكـاربون (^{1}H-^{13}C-NMR) اضافة إلى تحليل العناصر.

تم استخدام الصبغة المستخلصة من العنب كدليل في التحليل الكمي الحجمي وخاصة في تـسحيحات الحامض – القاعدة وذلك لدقتها الجيدة بالمقارنة مع الدلائل المعروفة مثل مثيل البرتقالي ومثيل الأحمـر والفينولفثالين. كما تم استخدامها في تحضير ورقة الدليل.

INTRODUCTION

There are different species of grape in the world, and there are some variations in each species, almost all grapes produced for wine, juice, jams, raisins, or table use are either of European or North American origin (Toni, 1998).

There is a great interest in plant polyphenols by different workers who are interested in these compounds. Polyphenol compounds include a considerable range of substances that differ in the number of carbon atoms in conjugation with the main structure of polyphenol combined with a number of hydroxyl group at different position (Debora and Kevin, 2002).

In grape pomace, a new pigment is detected by electrospary ionisation mass spectrometry (Fulcrand et al., 1998). The main products were identified using nuclear magnetic resonance (NMR) and HPLC.

Another pigment has been separated from Cabernet Sauvignon grape by paper chromatography and identified as malvidin. Two glycosides of malvidin are known, a petunidine glycoside, a delphinidin glycoside, and a complex diglycoside of malvidin (Bockian et al., 1955).

The scope of this work is to extract a new pigment from Grape (*Vitis vinifera L.*), which is used as indicator in the acid-base titration.

The purification of this pigment was done by different chromatographic techniques such as Thin-layer chromatography (TLC), Preparative thin-layer chromatography (PTLC), Paper chromatography (PC), Column chromatography, and High performance liquid chromatography (HPLC).

The identification of this pigment was established by different techniques like infrared spectroscopy, ^{1}H-nuclear magnetic resonance, ^{13}C-nuclear magnetic resonance, and ultraviolet spectroscopy.

EXPERIMENTAL

Materials and Reagents:

- Chemicals:

  All chemicals and reagents were of analytical grade and deionized water was used throughout.

- Glassware:

  Glasswares were heated at 600ºC for 12hr. before use to eliminate the contamination.

- Polyphenol Compound:

  The only polyphenol (National Vitamin), used as a reference compound was Rutin.

- Apparatus:

  UV-Visible double beam spectrophotometer Cecil 9000, was used with using quartz cell (1 cm).

Pye Unicam SP-3005 automatic and computerized IR-spectrophotometer was used during this work, in the range of 600-4000 cm^{-1}.

Bruker (300 MHz) instrument was used (from Glasgow University-UK) to obtain ^{1}H-NMR spectra.

A Bruker AMX500 ^{13}C-NMR was used (from Glasgow University-UK) for identification.

Exeter analytical model CE-440 was used to calculate the percentage of C, H and O content.

The HPLC system, consisted of a pump type water model 501 with a high pressure range 0-6000 psi (0-40 bars), was employed. The ZORBAX C_{18} (25 cm 4.6 mm i.d.) stainless steel column were used in this study.

Other Apparatus were Used Like:

Ultrasonic bath (Decon FS200) which used for degassing solutions in HPLC.
Rotary Evaporator: Laborota 4000 under vacuum, Heidolph Company (UK).
PH-meter (Model Jenway 3305) (S. Korean).
Furnace (model muffle furnace >600ºC) (USA).
Oven (model KOTTERMANN < 300ºC) (USA).
Press (model Pye Unicam) (USA).
Melting point apparatus (Ger) (Electrothermal 9100).
Electrical grinder (model Thomas).

Sampling:

The black grape was taken as a class of Iraqi Kurdistan grape in Hawler market (Type Koshnawate) in September-2002.

Two forms of samples in this research were taken, the first type is fresh grape, cleaned with water. The other type of sample was dry grape (Muwaz) treated directly without any treatment.

Determination of λ_{max}:

Dilute solution (1:1) of grape was prepared without any treatment from the crude fruit, then absorbance of the red solution in the range 350-750 nm was taken using UV-Visible instrument. The λ_{max} was 522.5 nm.

Isolation of the Red Pigment from Grape:

Six samples were taken of each type of grape (fresh and dried) (100-150 g) in plastic bottles and water was added (300 ml) for fermentation, this process takes (10-40 days) at room temperature (ranged between 24-27ºC). All samples were filtrated using filter paper (S and S No. 598), The filtrate was treated with 200 ml of 2% lead acetate for pigment precipitating (Gary, 2003). The mixture was transferred to a stopper flask and shaking very well for 3 min., stand for one hour. Finally separate the precipitate by filtration.

Washing the Isolated Pigment:

(1 g) of produced precipitate was treated for more than one continuous washing processes. First washed with (50 ml) water for at least three times, and then washed with (50 ml) diethylether. Finally washed with 30 ml of ethyl acetate (Harborne, 1981).

Purification of the Isolated Pigment:

The remain precipitate was treated with mixed solvent acetone: HCl (5:1), since, this mixed solvent is specific for anthocyanin extraction (Harborne, 1981). Table (1), shows the choice of mixed solvent. Chloroform, ethaylacetate and n-hexane give negative results.

Table 1: choice of mixed solvent for extraction of pigment.

Mixture solvent	**Results**
HCl (0.5N): Chloroform	-
HCl (0.5N): Ethyl acetate	-
HCl (0.5N): n-Hexane	-
HCl (0.5N): Acetone	+
HCl (0.7N): Acetone	+
HCl (1N): Acetone	++
HCl (1.5N): Acetone	++
HCl (2N): Acetone	+++
HCl (2.5N): Acetone	+

(-) mean there is no extraction, (+), (++) and (+++) gives good conditions for extraction especially (+++).

The mixture was transferred to a stopper flask, shaked for 10 min., and stood for (1 hour). The filterate and precipitate were separated using a filter paper (S and S No. 598). The samples were concentrated at 40ºC using rotary evaporator. Finally, the concentrated solution dried at room temperature in a dark place to obtain red solid material (Gary, 2003).

Moisture Determination:

The water content is frequently an index of stability and quality, and is also a measure of yield and quantity of food solids (Betty et al., 2002).

(2 g.) of the red pigment was weighted in crucible, which had been previously dried in a hot air oven and cooled in a desiccator. Placed in a hot air oven and dried over (1 hour) intervals at a temperature of 100º-102ºC. It was removed and cooled to room temperature in a desiccator and weighted again. This process was repeated five times till the lost in weight become constant (Bruce et al., 2002).

Anthocyanin Test:

Dry grape samples were heated in (3-4 ml) 2M HCl in a test tube for 40 min, at 100ºC. The colored extract is cooled and decanted from the plant tissue. The cooled extract was washed twice with ethyl acetate to remove any flavones if present (Harborne, 1981), the ethyl acetate layers being discarded and the remaining aqueous layer being heated at 80ºC for 3 min., to remove the last trace of ethyl acetate. The pigment was then extracted into a small volume of amyl alcohol, which can be pipetted off and concentrated to dryness by heating on a water bath. The anthocyanin in the residue was dissolved in 2-4 drops of methanolic HCl (1% HCl in methanol) and chromatographed in one dimensional on paper chromatography in Forestal (HCl: Formic acid: H_2O 2:5:3) and formic acid solvents.

Determination of Sulfur:

The samples were tested for sulfur content using Gibbs apparatus (treatment of sodium sulfide with concentrated sulfuric acid) (Vogel, 1961).

Chromatographic Techniques for Purification:

TLC: Different solvent mixtures were used as a mobile phase in TLC for testing the red solid extracted material on glass plate, coated by silica gel.

PTLC: The TLC plate was air-dried for (1 hour) and activated in an oven at 115ºC for (4 hours) prior to use. (0.1 g.) of red pigment was dissolved in 10 ml of methanol (10 mg/ml), then using capillary tube to make a small spots of the solution in more concentrated zone on their bottom of plate (2 cm from the bottom) as thick line. The plate was placed in a TLC tank with different mobile phases for at least (3 hours).

The silica gel was removed which surrounded each of spots after development under UV-light with small spatula and dissolved in methanol, then the mixture was filtered to remove all silica gel from the solution. The mixture is concentrated with vacuum rotary evaporator at 60ºC. The residue was placed in a watch glass and stands for (2 hours) to obtain the solid material (Jow and David, 1998).

PC: Different solvent mixture used as mobile phase for testing the red sample on the sheets.

HPLC: (6.80 g.) of KH_2PO_4 dissolved in small amount of water then completed to 1L with 20% methanol to prepare organic modifier with 0.05M KH_2PO_4 as buffer. (0.1 ml.) of red pigment solution which prepared by dissolving (1 g.) of pigment in methanol was taken, and injected for analysis.

Identification of the Red Pigment:

Red pigment which was extracted from grape sample was identified using different techniques, IR-, UV-, ^{1}H-NMR and ^{13}C-NMR spectroscopy, also CHN analysis was used to detect the percentage of C, H and O content. Other identification tests were also done such as ignition, melting point and solubility.

RESULTS AND DISCUSSION

Isolation the Red Pigment:

(2.5 g.) of red pigment was isolated from (100 g.) grape sample (fresh), (2.5%). The isolated compound was red solid non-crystalline material,. Table (2), shows the physical and chemical tests for isolated pigments.

The isolated pigment gives two spots by TLC and PC which indicate that the extracted substance containing two different compounds. Table (3), shows the TLC results using different developers. The quantity present of the isolated product in different experimental conditions (temperature, pH and time) were recorded. Tables (4), (5), and (6), show the results. From these results it seems that the best conditions for this isolation takes place at 70ºC, pH = 3.0 and (3.0 hours) time of extraction. This indicates that above and below these values the percentage of isolated pigment decreases where high temperature and high pH may cause desolution of the isolated pigment, while the extraction for a long time having the same effect.

Table 2: Physical and chemical tests for isolated pigment from grape.

Tests	Results
Physical State	Solid, non-crystalline, red (blackish)
Melting point	Decomposed at 190-200ºC
Ignition test	Fume black results some carbonization
Solubility test	Soluble in water, methanol and ethanol
Odor	Odorless
Carbohydrate	Violet ring
Glycoside	Red precipitate

Table 3: TLC results for isolated pigment from grape.

Reagent	Results	R_f	Notes
UV-light	+ve	0.47,0.60	Conjugated double bonds
Pure eye	+ve	0.46, 0.62	Anthocyanin content
Sulfuric acid 4%	+ve	0.45,0.63	Glycosides, sugar attached
Iodine	+ve	0.46,0.62	unsaturated organic compound
Ammonium hydroxide 10%	+ve	0.45,0.61	Anthocyanin content
Lead acetate 1%	+ve	0.45,0.60	Anthocyanin content
Formaldehyde	+ve	0.45,0.60	poly aromatic hydrocarbon
Alcoholic aluminum chloride (5%)	+ve	0.46, 0.60	Unsaturated cyclic compounds contain oxygen atom
p-ansialdehyde	+ve	0.45,0.60	Carbohydrate content

Table 4: Effect of temperature on quantity of isolated red pigment (at pH = 3.5; time of extraction = 3 hours).

Temperature (ºC)	(%) of isolated pigment
50	0.65
60	1.42
70	2.52
80	2.20
90	1.63

Table 5: Effect of pH on quantity of isolated red pigment (Temp. 70ºC; time of extraction = 3 hours)

pH	(%) of isolated pigment	pH	(%) of isolated pigment
1	1.86	6	1.62
1.5	2.01	6.5	1.58
2	2.23	7	1.35
2.5	2.59	8	0.98
3	2.51	9	0.61
3.5	2.48	10	0.55
4	2.26	11	0.41
4.5	2.20	12	0.25
5	2.01	13	0.19
5.5	1.95		

Table 6: Effect of time of extraction on quantity of isolated red pigment (Temp. 70ºC; pH = 3)

Time (hr.)	(%) of isolated pigment
1	1.02
2	1.5
3	2.50
4	2.56
5	2.54
6	2.51
10	2.52
14	2.50
18	2.50
24	2.51
36	2.50
48	2.53
60	2.52
72	2.49

Determination of Water, Sulfur and Essential Oils:

The quality of the Iraqi Kurdistan grape was examined with respect to water, sulfur and essential oils contents. Table (7), illustrates these results. The results obtained were acceptable as compared with the standard values of grape (Muntean et al, 1997).

Table 7: Some tests for the grape sample.

Tests	Results	Standard values [12]
Water Content	74.2%	80%
Sulfur	-ve	-ve
Essential oil (ml/Kg)	+ve (0.27)	+ve (0.3)

Table 8 : Results of isolated pigment with PC.

Mobile phase (ratio)	R_f
HCl: Acetic acid : H_2O (3:30:10)	0.50, 0.61
HCl: Formic acid: H_2O (2:5:3)	0.24, 0.28
n-Butanol: Acetic acid: H_2O (4:1:5)	0.71, 0.55

Chromatographic Techniques for Identification:

Thin-Layer Chromatography:

For the purification of red pigment which was isolated from the grape, TLC plate was used, The same developers illustrated in Table (2), were employed to detect the R_f values for the two spots. All of mobile phases and developers showing two spots for two components in the sample.

Preparative Thin-Layer Chromatography:

This method was applied for separation of the two constituents in the red pigment isolated from the grape. The first constituent was identified as cyanidin and the second as malvidin depending on the R_f values on the TLC comparing with standards (Harborne, 1981).

Paper Chromatography:

The results of two spots were shown in Table (8), using different mobile phases. The two spots of magenta color for anthocyanin compound and purple color for another anthocyanin compound were detected.

High Performance Liquid Chromatography (HPLC):

The isolated red pigment from the grape was tested by HPLC technique. The qualitative analysis of the red pigment showed the presence of two polyphenols by comparing their retention time (t_R) with that for standard polyphenol as a reference compound. (Fig. 1a), represents the chromatogram of red pigment. The chromatogram shows two peaks representing two compounds with different retention times. The first compound has retention time (t_R) 3.52 min and the t_R for the second compound was 6.00 min. (Fig. 1b), shows the chromatogram of the polyphenol reference compound which showed retention time (t_R) 3.41 min.

The analysis of samples by HPLC must be compared with standard materials, but unfortunately do not get any polyphenol compounds except Rutin.

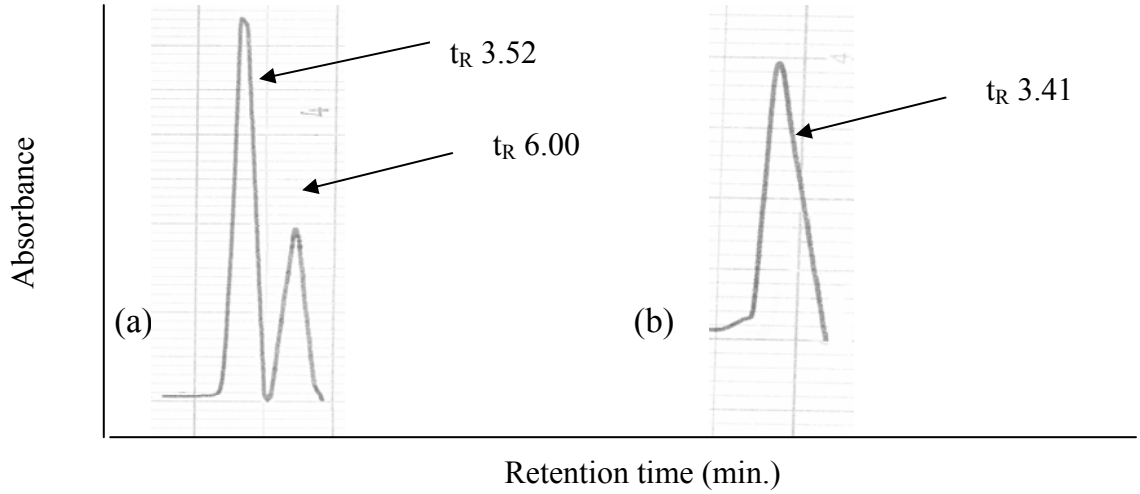


Fig. 1: HPLC chromatogram using (ZORBAX C_{18}) column:
(a) For red isolated pigment
(b) For reference polyphenol compound

Identification of the Red Pigment:

In addition to different chromatographic techniques as mentioned above which were used for identification of the product pigment, other techniques such as I.R., U.V. ^{1}H-NMR, ^{13}C-NMR and CHN analysis were also employed. IR-spectrum for the first compound, showed bands at 1640 cm^{-1} for C=C of benzene ring, 1723 cm^{-1} for carbonyl group, 2967cm^{-1} for aliphatic hydrogen, 3032 cm^{-1} for aromatic hydrogen, and 3429 cm^{-1} for –OH group.

IR-spectrum for the second compound showed approximately the same bands, due to the similarity in the functional groups of the two isolated compounds.

The UV spectrum for the isolated red pigment from the grape sample. The spectrum showed absorption band at 273 nm. This is similar to those of anthocyanin compounds.

The ^{1}H-NMR spectrum showed the following signals for protons in the compound isolated from the grape, band at 3.38 ppm corresponding to methine proton in the sugar molecule attached the aglycone at position three, 4.16 and 4.69 ppm also corresponding to methylene groups for the same molecule. Peaks at 5.2-6.5 ppm corresponding to the aromatic protons in the main molecule. Peak at 6.9 ppm corresponding to the –OH protons in the molecule, while the band at 3.73 ppm corresponding to methyl protons in the molecule.

The ^{13}C-NMR spectra of isolated compound, shows the peak at 56.6 ppm for methoxy carbon in the molecule, peaks at 64.4, 67.9 and 73.7 ppm for carbon atoms in fructose molecule. The results of elemental analysis were illustrated in Table (9), There are very closeness and similarity between the theoretical and experimental values.

Table 9: Elemental analysis of isolated pigment.

Extracted compound Malvidin-3-fructoside	**Theoretical Value**			**Experimental value**		
	C%	**H%**	**O%**	**C%**	**H%**	**O%**
Red pigment	55.87	5.30	38.83	55.95	5.45	38.60

Anthocyanin Tests:

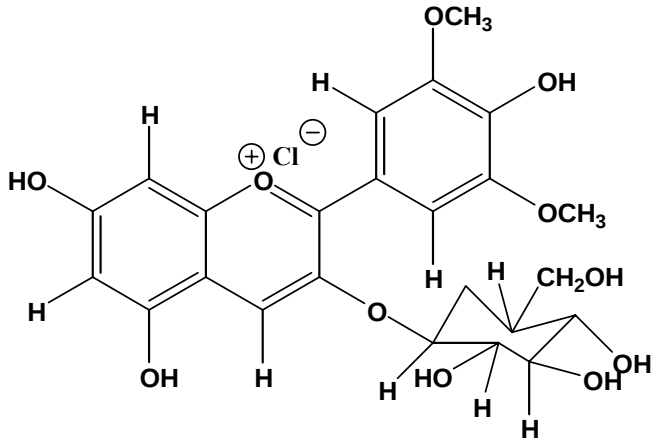
These tests were done on the red pigment before and after hydrolysis. Table (10), shows the results of TLC before and after hydrolysis using Forestal and (n-butanol: acetic acid:H_2O (4:1:5) (BAW) as mobile phase and UV light for detecting the spots. Results show that anthocyanidin compound (malvidin) was identical with the results of the standard, in contrast the hydrolysis only malvidin-3-glycoside was also gives identical results compared with standard.

Table 10: TLC results before and after hydrolysis**.**

Pigment	R_f before hydrolysis		R_f after hydrolysis
	Forestal	BAW	BAW
Isolated red	0.61	0.57	0.37

Structure of Red Pigment:

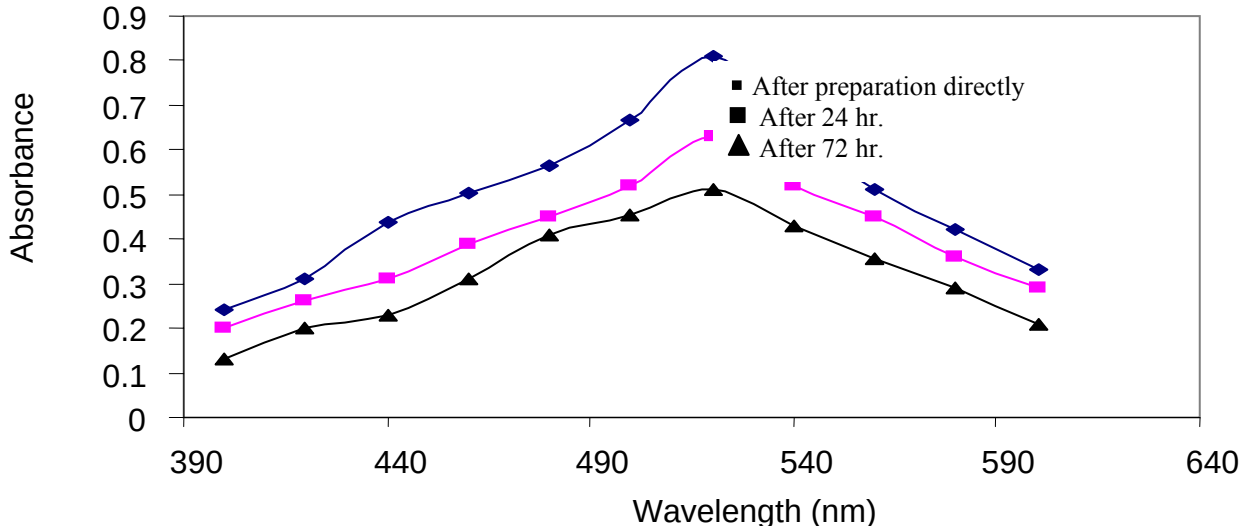
Results obtained from different techniques which were used here to identify the structure of red pigment, indicating that it is Malvidin-3-fructoside. Depending on the R_f values in standard tables for natural pigments (Harborne, 1981). The structure of compound was confirmed by comparison of its UV spectrum and ^{1}H-NMR to the known compound Malvidin-3-fructoside in addition to values of R_f for TLC, PTLC and PC. Accordingly the structure of the red pigment may be given as follows:



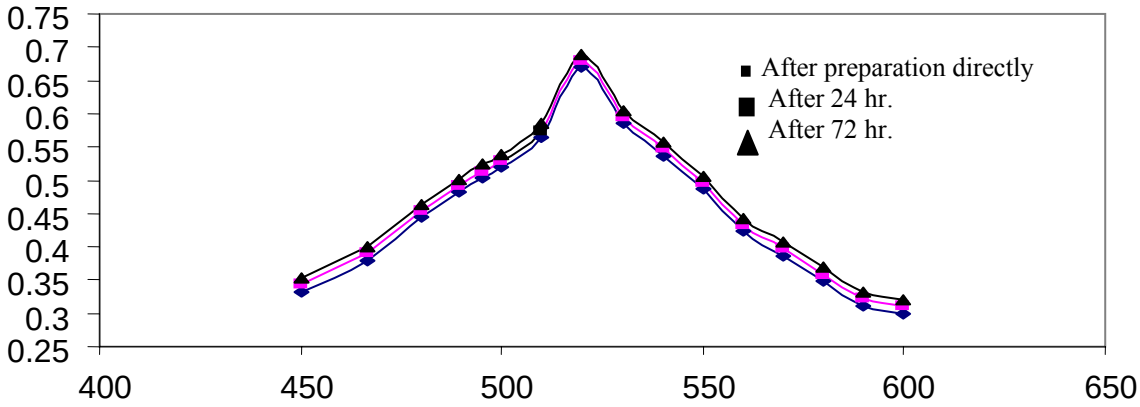
APPLICATIONS

Validity of the Red Pigment as Indicator:

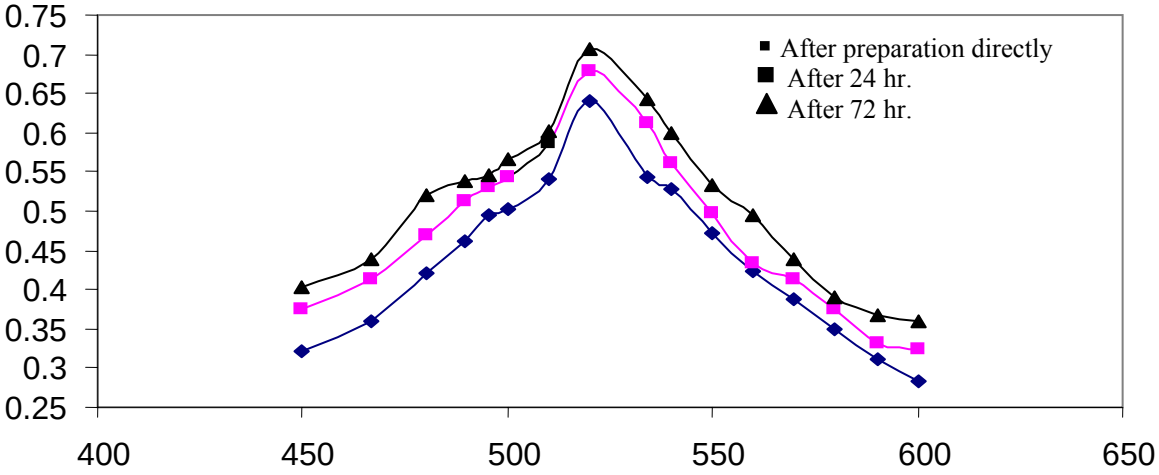
Different studies such as solvent effect, temperature effect and effect of pH on the color stability of pigment were done. The results show that the best solvent for the pigment when used as indicator was methanol or ethanol, because the λ_{max} does not change during different times, (Fig. 2).



a) Water



b) Methanol



c) DMSO

Fig. 2: Spectra of red pigment using water, methanol and DMSO as solvents.

The effect of temperature on the stability of the pigment was studied. (Fig. 3), shows that there was no effect of temperature up to 80ºC on the pigment stability because the anthocyanin compounds are known to decompose at a temperature higher than that used in this study.

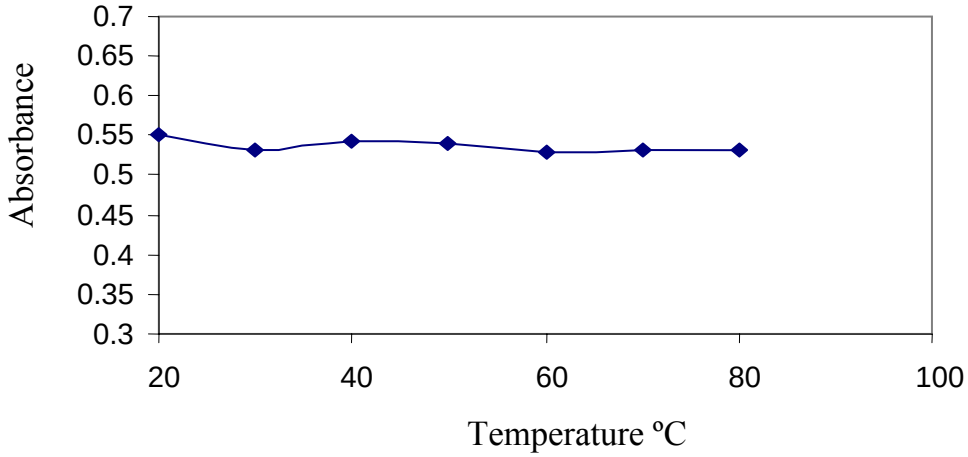


Fig. 9: Effect of temperature on the stability of pigment.

Finally, the effect of pH on the color stability of pigment was studied. It was shown that the color of pigment was changed to pink at lower pH. Therefore, the color and stability of the pigment was highly dependent on the pH.

Determination of pKa:

As part of our investigation, the proton dissociation of the pigment was studied by potentiometric titration of the pigment solution (0.25 g/100 ml H_2O) with 0.1M NaOH. A plot of pH vs. volume of NaOH showed one distinct dissociation region. The proton dissociation constant (pKa) determined to be 6.21. The magnitude of Ka indicates the tendency of the hydrogen atom to be ionized. The value of Ka shows that the pigment is a weak acid (the smaller the value of Ka, the weaker is the acid).

Comparison of Red Pigment with Common Indicators:

The results show that the amount of NaOH solution (0.1M) required to neutralize 10 ml of 0.1M HCl was 10.6, 11.0, 10.12 and 11.2 ml when using isolated pigment, methyl orange, methyl red and phenolphthalein as indicators respectively.

Recommended Quantity of Indicator:

Different quantities of indicator (1-4%) of pigment solution were used. It was found that 3% was enough to give a sharp end point in acid-base titration with volume (1-2 ml).

Precision and Accuracy of the Method:

As shown in Table (11), the precision for titration of 10 ml of 0.1N standard HCl against 0.1N sodium hydroxide (n= 3) was found to be 0.16% using malvidin-3-fructoside pigment as indicator. Table (12), shows the relative error for the pigment which compared with common indicators. These results indicate a good precision and accuracy.

Table 11: Precision of titration of 10 ml of 0.1N HCl against 0.1N NaOH using malvidin-3- fructoside as indicator.

No.	E.P.	D=(X-X′)	D^2	S.D.	C.O.V.	RSD%
1	10.62	0.024	0.000576	0.0170	0.0016	0.160
2	10.58	-0.016	0.000256			
3	10.59	-0.006	0.000036			

Table 12 :Accuracy of the pigment.

No.	Common indicators	Er %
1	Methyl orange	3.87
2	Methyl red	-4.5
3	Phenolphthalein	4.8

CONCLUSION

The pigment of a grape fruit was suggested as very useful indicator in visual acid-base titration in aqueous medium, according to the good precision and accuracy of the method. Comparing with common indicators such as methyl orange, methyl red and phenolphthalein. The present indicator was very cheap and its extraction was simple compared with other natural indicators in acid-base titration.

REFERENCES

Betty, E.M., 2002. World's Women Congress; Chemistry Department, Makerere University, Kampala-Uganda.

Bruce, R.D., Geoff, H. and Ian, B., 2002. Food Chemistry A Practical Manual, University of Queensland Publication. pp.2-3.

Bockian, A.H., Kepner, R.E. and Dinsmoor Webb, A., 1955. Wine Production; 3(8), pp.695-699.

D'Elia, C.F., 1987. Report Prepared for Chesapeake Bay Program Liaison Office, Region III, US EPA.

Debora, T. and Kevin, R., 2002. Sample Handling Strategies for the Determination of Biophenols in Food and Plants. Journal of chromatography A, pp.975, 71-93.

Fulcrand, H., Benabdeljalil, C., Rigaud, J., Cheynier, V., and Moutounet, M., 1998. A New Class of Wine Pigments Generated by Reaction Between Pyruvic Acid and Grape Anthocyanins**.** Phytochemistry; 47(7), pp.1401-1407.

Gary, W., 2003. Polyphenol Extraction from Foods. Ch.1, pp.7-25.

Harborne, J.B., 1981. Phytochemistry Methods, pp.56-57.

Jow, W. and David, W., 1998. "Biochemistry" 321, Exp.10".

Muntean, D. and Csedo, C., 1997. International Conference on Medicinal and Aromatic Plants (part II); ISHS Acta Horticulturae. 597 p.

Toni, S., 1998. Hefl@psu.edu or Webmaster (Txs26@psu.edu).

Vogel, A.I., 1961. A Text Book of Quantitative Inorganic Analysis, 3rd Edition, Longmans, London, 524 p.