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Hippeastrum hybridum anthocyanins as indicators of endpoint in acid – base titrations

Robert BYAMUKAMA[1*], Eric Nicholas WANINDA[1], David MUKAMA[1] and Jane NAMUKOBE[1]

[1]*Department of Chemistry, School of Physical Sciences, College of Natural Sciences, Makerere University, P.O. Box 7062, Kampala, Uganda.*

[*]*Corresponding author; E-mail: rbyamukama@cns.mak.ac.ug, Tel.: +256-392548585 ; Fax: +256-41-531061*

ABSTRACT

Anthocyanins from *Hippeastrum hybridum* (Amaryllis) were investigated as indicators of endpoint in acid- base titrations. Extraction of the anthocyanins was done using distilled water, methanol and methanol containing 0.5% acetic acid. The extracts were used in determination of endpoint in titrations between strong acid/strong base, strong acid / weak base, weak acid / strong base and weak acid / weak base at concentration levels of 0.1 M, 0.5 M and 1.0 M. The titres were compared to those obtained using phenolphthalein, methyl orange and mixed indicator. The shelf life of anthocyanin extracts kept in amber bottles at room temperature (16.8 ^{0}C – 29.1 ^{0}C) was also investigated. The plant extracts gave accurate and precise results in titration of strong acids with strong bases and weak acids against strong bases at all concentrations investigated (0.1, 0.5 and 1.0 M) that were very comparable to the values obtained using phenolphthalein, methyl orange and mixed indicator. In titration of weak acids against weak bases, the plant extracts gave accurate results when concentration was 0.1 M but failed for 0.5 M and 1.0 M. On average the anthocyanins from *Hippeastrum hybridum* could be used in the titration of strong acid versus strong base, weak acid versus strong base and weak acid versus weak base. The anthocyanins from *Hippeastrum hybridum* had a good shelf life of 90 days when kept at room temperature. The extracts can thus be used in any place including those that have no access to refrigeration facilities since they can be kept at room temperature for some time before they undergo degradation.

Keywords: *Hippeastrum hybridum,* anthocyanins, pH indicators, acid-base titrations, shelf life

INTRODUCTION

Volumetric analysis is one of the most commonly used quantitative techniques in chemical analysis in a wide range of fields and indicators are used to show endpoints. An indicator is a weak organic acid or base that changes color when the pH in the medium changes. The continued use of synthetic indicators has raised concerns on their safety to the users and the environment in addition to the cost especially to the developing world who import them and have no proper chemical disposal strategies in place and can easily contaminate the public drinking water system (Cooper et al., 2004; Dunnick, 2008; Eze and Ogbuefi, 2014). Natural compounds from plants have been earmarked as potential alternatives to synthetic indicators.

Anthocyanins are colored compounds that have a potential to act as suitable alternatives to synthetic indicators (Abugri et al., 2012; Pojer et al., 2013). Anthocyanins just like synthetic indicators, are able to change colors with pH changes by donating or

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accepting protons (Torskangerpoll and Andersen, 2005). Gradually increasing the pH causes a gradual shift of anthocyanin colors from red towards blue tones (Torskangerpoll and Andersen, 2005). Anthocyanin based indicators have a possibility and advantage of being prepared just before the experiment and their color changes at endpoints are sharp (Bhagat et al., 2008; Kadam et al., 2013; Marulkar et al., 2013). The use of anthocyanins as natural indicators is safe as they are non-toxic and environmentally compatible (Osabohien, 2014) in addition to having other uses (Dao et al., 2014; Toni and Djossa, 2015).

The challenge of instability and absence of standard formulations in place limit the application of anthocyanins as indicators of endpoints in acid-base titrations. The stability of anthocyanins is affected by a number of factors which include pH, temperature, structure, concentration, light, oxygen, solvents, hydration, presence of enzymes, co-pigmentation as well as presence of metallic ions (Gradinaru et al., 2003; Bordignon-Luiz et al., 2007; Mpiana et al., 2012). Anthocyanins from different sources have varying stabilities and addition of sugars and salts increases instability (Rosso and Mercadante, 2007). The acylated and methylated anthocyanins have been found to be more stable compared to the unacylated or un methylated counterparts (Torskangerpoll and Andersen, 2005; Wu and Prior, 2005).

We previously reported anthocyanins from flowers of *Hippeastrum hybridum*, a perennial ornamental plant belonging to the family Amaryllidaceae (Byamukama et al., 2006). The genus *Hippeastrum* has about 90 species and over 600 hybrids and cultivars (Alexandre et al., 2011). *Hippeastrum hybridum* grows in tropical and semi tropical regions where it can be planted all year round (De Bruyn, 1997). In the present study, anthocyanins from *H. hybridum* were explored as potential substitute to synthetic indicators in titration of acids against bases at varying concentrations. The shelf life of anthocyanin indicators from *H. hybridum* was also established over a period of 90 days when stored at room temperature.

MATERIALS AND METHODS

Collection of plant materials

The *Hippeastrum hybridum* cv. spp. flowers were collected from flower garden between the Mathematics Department Building and Institute of Environment and Natural resources at Makerere University. The samples were taken to the herbarium of Botany Department, Makerere University for identification. A voucher specimen (Mukama No.3/2014) was deposited in the herbarium of the same department. The collected samples were weighed and kept in deep freezer before extraction.

Extraction and analysis to confirm presence of identified anthocyanins

The flowers of *Hippeastrum hybridum* (68 g) were cut into small pieces and then soaked in 200 ml of methanol containing 0.5% acetic acid in a conical flask. The flask was covered using parafilm and left to stand in a deep freezer for 48 hours. The filtered extract was concentrated under reduced pressure at 29 °C using rotary evaporator. The concentrated sample was partially purified by solvent extraction (several times) using ethyl acetate after which it was applied to amberlite XAD-7 column and washed with distilled water until the eluent was neutral to litmus paper. The purified anthocyanins were desorbed from the column by elution using methanol containing 0.5% acetic acid, concentrated and carefully loaded onto the column containing sephadex LH-20 ion exchange resin. The anthocyanins were eluted from the column using a 30% aqueous methanol containing 0.5% acetic acid. The above extraction procedures have been used previously by Byamukama et al. (2006). The fractions containing anthocyanins were collected according to the visual band separations and concentrated on the rotary evaporator.

Co-chromatography (TLC, online HPLC)

TLC analysis was carried out on fractions from sephadex LH-20 column using, microcrystalline cellulose (F 5565, Merck) with the solvent FHW (Formic acid: HCl: Water; 1:1:2). Analytical HPLC was carried

out an HP-1050 Model system (Shimadzu) using an ODS column, C_{18}, (4.6×250 mm, 5 µm). Solvent A (Phosphoric acid-water 1.5:98.5; v/v), and solvent B (phosphoric acid-acetic acid- acetonitrile-water 1.5:20:25:53.5; v/v) were used. The elution profile consisted of isocratic elution (20% B) for 5 minutes, linear gradient from 20% to 55% during the next 10 minutes, linear gradient from 55% to 90% during the next 30 minutes, isocratic elution (90%) for 2 minutes and finally a linear gradient from 90% to 20% for 3 minutes. The flow rate was 1 ml/min and the diode array detector (DAD) was set to monitor anthocyanins at 520 ± 20 nm (Andersen and Francis, 2004). Anthocyanins from *Hippeastrum hybridum* were co-chromatographed with anthocyanins from black currant (*Ribes nigrum*) (Frøytlog et al., 1998).

Extraction and determination of endpoint

The flower samples of *Hippeastrum hybridum* (5.608 g, 5.123 g and 5.353 g) were separately crushed with a pestle in a mortar and then dissolved in 30 ml of distilled water, methanol and methanol containing 0.5% acetic acid in separate flasks respectively (Patil et al., 2009). The extracts were each filtered through a filter funnel containing Whatmann no.4 filter paper (110 mm) to remove any solid particles. The three filtrates were separately covered with parafilm and kept in deep freezer prior to use on the same day. All the titration experiments were carried out at room temperature. 10 ml portions of 1.0, 0.5 and 0.1 M of the acid concentrations were respectively titrated against 1.0, 0.5 and 0.1 M solutions of base with 5 drops of the anthocyanin indicator for each of the anthocyanin solutions extracted. The acids used were Hydrochloric acid (HCl), acetic acid while the bases were sodium hydroxide (NaOH) and ammonia solutions. For comparison purposes, the experiments were repeated by using 3 drops of phenolphthalein indicator in the titration of strong acid (aqueous HCl) against strong base (aqueous NaOH), methyl orange for strong acid versus weak base (Ammonia solution) and the mixed indicator in the titration of weak acid (acetic acid) versus weak base (ammonia solution). The mixed indicator was prepared by mixing neutral red with methylene blue in the ratio of 2:3 and dissolving it in ethanol (Harris, 2010; McGuire, 1941).

Data Analysis

The average titre values obtained using the anthocyanin extract were compared with the ones obtained using phenolphthalein, methyl orange and mixed indicator by the student t-test. The t value less than the critical t value (t_c) indicated that the values were not significantly different at 95% confidence interval while t value greater that the t_c indicated that the values were significantly different (Driscoll and Lecky, 2001).

Experimental determination of shelf life of extracted anthocyanins in liquid form at room temperature

The flower sample of *Hippeastrum hybridum* (51.222 g) was crushed with a pestle in a mortar and then dissolved in 100 ml of methanol containing 0.5% acetic acid in a flask. The extract was filtered through a filter funnel containing Whatmann no.4 filter papers (110 mm) to remove any solid particles. The filtrate was divided into two portions which were put in two separate corked amber bottles keeping one in a deep freezer and the other on an open shelf (Contreras-Lopez et al., 2014). 10 ml portions of 0.1 M hydrochloric acid were titrated against 0.1 M sodium hydroxide solution with 5 drops of the anthocyanin extracts kept in amber bottles. The experiment was repeated four times to get four titre values. For comparison purposes, the experiments were repeated by titrating 10 ml of the titrant with 3 drops phenolphthalein indicator. The procedure was repeated after 1 day, 3 days, 5 days, 10 days, 21 days, 30 days, 60 days and 90 days.

RESULTS

Identification of anthocyanins from *Hippeastrum hybridum*

The anthocyanins from *Hippeastrum hybridum* were first confirmed by the Thin Layer Chromatography (TLC) and High

Performance Liquid Chromatography (HPLC). They were co-chromatographed with anthocyanins from black currant (*Ribes nigrum*) (Frøytlog et al., 1998).

TLC analysis on the crude extract and fractions from sephadex LH-20 showed two anthocyanins in the flower extracts of *Hippeastrum hybridum* (Table 1). The HPLC analysis (detection at 520 ± 20 nm) on the fractions from sephadex LH-20 also showed presence of two anthocyanins in the flower extract of *Hippeastrum hybridum* (Table 1) which was in agreement as previously reported by Byamukama et al. (2006).

Titration of aqueous HCl versus aqueous NaOH

In the titration of aqueous HCl against aqueous NaOH, the anthocyanins extracted from *Hippeastrum hybridum* gave endpoints which were in the same range as those given by phenolphthalein at all concentration levels (0.1 M, 0.5 M, 1.0 M) investigated (Table 2). The acid concentrations calculated from average titres obtained using extracts of *Hippeastrum hybridum* were similar to those calculated from average titres obtained when using phenolphthalein (Table 2). The student t-test analysis of results showed that, at 95% confidence interval, there was no significant difference between titres obtained using anthocyanins of *Hippeastrum hybridum* and titres obtained using phenolphthalein ($t < 3.182$). The precision of the anthocyanin indicator which was measured by determining their coefficient of variation (CV) values was in the same range as that of phenolphthalein indicator (Table 2).

Aqueous HCl versus Ammonia solution

In the titration of aqueous HCl versus ammonia solution at all the concentration levels (0.1 M, 0.5M and 1.0 M) using anthocyanins extracted from *Hippeastrum hybridum,* the titre values obtained using the extracts as indicator were slightly higher from those obtained when using methyl orange (Table 3). The calculated acid concentrations from titres by the extracts were also slightly higher than those obtained from titres by methyl orange (Table 3). The student t-test analysis of the results revealed that at 95% confidence interval, only the methanol extract (unacidified) ($t < 3.182$) gave the titre values that were similar to those by methyl orange indicator when concentration was 1.0 M (Table 3).

Aqueous acetic acid versus aqueous NaOH

The titre values obtained when using anthocyanins extracted from flowers of *Hippeastrum hybridum* in the titration of aqueous acetic acid against aqueous sodium hydroxide were generally similar as those obtained using phenolphthalein for all concentrations (Table 4). The concentrations of the acid calculated using titres obtained using anthocyanins of *Hippeastrum hybridum* were similar to the one obtained when titres by phenolphthalein were used. The student t-test analysis of results showed that at 95% confidence interval, the titres by *Hippeastrum hybridum* were generally similar ($t < 3.182$) to titres obtained while using phenolphthalein as an indicator (Table 4). The precision by the anthocyanin extracts of *Hippeastrum hybridum* was not very different from that of phenolphthalein according to the coefficient of variation values (Table 4).

Aqueous acetic acid versus ammonia solution

In the titration of acetic acid, versus ammonia using anthocyanins extracted from *Hippeastrum hybridum*, the titres obtained by the methanolic anthocyanin extracts were similar to those by the mixed indicator when the concentration of reagents was 0.1 M but different when concentrations were 0.5 M and 1.0 M (Table 5). The student t-test analysis showed that at 95% confidence interval the acidified methanol and unacidified methanol generally gave titres that were statistically similar ($t < 3.182$) to those by mixed indicator when the concentration of reagents was 0.1 M (Table 5).

Determination of shelf life of *Hippeastrum hybridum* extract kept at room temperature

The titre values obtained by anthocyanin extracts kept on open shelf at room temperature were compared with titres

obtained by the anthocyanin extracts maintained in a deep freezer as well as titres by phenolphthalein. Average titres and acid concentration of the same solution were calculated over a period of 90 days. The *Hippeastrum hybridum* extract maintained its color and consistently gave accurate results for all the 90 days it was kept at room temperature on an open shelf (Table 6). The calculated acid concentration was the same throughout the entire period (90 days) and was similar to the concentrations got for the extracts kept in deep freezer and those obtained using phenolphthalein (Table 6). The precision with which the extract gave titres over the entire period was similar which shows the ability of the anthocyanins from *Hippeastrum hybridum* to consistently show correct endpoints in acid base titrations.

Table 1: Chromatographic (TLC, on-line HPLC) data recorded for anthocyanins extracted from flowers of *Hippeastrum hybridum.*

Compound	R_f (TLC), FHW	t_R (on-line HPLC) (min)
1	0.51	18.974
2	0.63	17.397
1[a]	0.49	18.727

1[a] Cyanidin 3-rutinoside from black currant (*Ribes nigrum*) (Frøytlog et al., 1998).

Table 2: The average titres obtained in the titration of aqueous HCl with aqueous NaOH using anthocyanin extracts of *Hippeastrum hybridum* flowers and phenolphthalein as indicators at different concentration levels.

Concentration of base used / M	Indicator used	Average titre±SD /ml	t-value (magnitude) (t_c=3.182)	Coefficient of variation / %	Calculated concentration /M
1.0	Water extract	10.05±0.058	1.000	0.574	1.005
	Methanol extract	10.03±0.096	0.000	0.955	1.003
	Acidified methanol extract	10.08±0.050	1.732	0.496	1.008
	Phenolphthalein	10.03±0.050	n/a	0.499	1.003
0.5	Water extract	10.08±0.050	1.732	0.496	0.504
	Methanol extract	9.98±0.050	1.732	0.501	0.499
	Acidified methanol extract	9.95±0.058	1.567	0.580	0.498
	Phenolphthalein	10.03±0.050	n/a	0.499	0.501
0.1	Water extract	10.03±0.050	1.000	0.499	0.1003
	Methanol extract	10.05±0.058	1.732	0.574	0.1005
	Acidified methanol extract	9.98±0.050	1.000	0.501	0.0998
	Phenolphthalein	10.00±0.000	n/a	0.000	0.1000

SD: Standard deviation, t_c: critical value of t, n/a: not available.

Table 3: The average titres obtained in the titration of aqueous HCl with NH_3 solution using anthocyanin extracts of *Hippeastrum hybridum* flowers and methyl orange as indicators at different concentration levels.

Concentration of base used /M	Indicator used	Average titre±SD /ml	t-value (magnitude) (t_c=3.182)	Coefficient of variation / %	Calculated concentration / M
1.0	Water extract	10.35±0.058	8.660	0.558	1.035
	Methanol extract	10.03±0.050	1.567	0.499	1.003
	Acidified methanol extract	10.35±0.058	5.000	0.558	1.035
	Methyl orange	10.10±0.082	n/a	0.808	1.010
0.5	Water extract	10.58±0.171	9.798	1.615	0.529
	Methanol extract	10.53±0.096	7.000	0.910	0.526
	Acidified methanol extract	10.73±0.150	19.053	1.399	0.536
	Methyl orange	10.18±0.096	n/a	0.941	0.509
0.1	Water extract	10.45±0.100	5.422	0.100	0.1045
	Methanol extract	10.48±0.096	15.000	0.096	0.1048
	Acidified methanol extract	10.50±0.141	9.798	0.141	0.1050
	Methyl orange	10.10±0.082	n/a	0.082	0.1010

SD: Standard deviation, t_c: critical value of t, n/a: not available.

Table 4: The average titres obtained in the titration of acetic acid solution with aqueous NaOH using anthocyanin extract of *Hippeastrum hybridum* flowers and phenolphthalein as indicators at different concentration levels.

Concentration of base used/M	Indicator used	Average titre±SD/ml	t- value (magnitude) (t_c=3.182)	Coefficient of variation / %	Calculated concentration /M
.0	Water extract	10.03±0.050	3.000	0.499	1.003
	Methanol extract	9.90±0.082	0.775	0.825	0.990
	Acidified methanol extract	9.95±0.058	0.000	0.580	0.995
	Phenolphthalein	9.95±0.058	n/a	0.580	0.995
0.5	Water extract	9.85±0.100	2.499	1.015	0.493
	Methanol extract	9.80±0.082	5.196	0.833	0.490
	Acidified methanol extract	9.73±0.096	3.576	0.985	0.486
	Phenolphthalein	9.95±0.058	n/a	0.580	0.498
0.1	Water extract	9.80±0.082	3.000	0.833	0.0980
	Methanol extract	9.85±0.058	2.449	0.586	0.0985
	Acidified methanol extract	9.90±0.082	1.73	0.825	0.0990
	Phenolphthalein	9.95±0.058	n/a	0.580	0.0995

SD: Standard deviation, t_c: critical value of t, n/a: not available.

Table 5: The average titres obtained in the titration of acetic acid solution with NH_3 solution using anthocyanin extract of *Hippeastrum hybridum* flowers and the mixed indicator as indicators at different concentration levels.

Concentration of base used /M	Indicator used	Average titre±SD /ml	t-value (magnitude) (t_c=3.182)	Coefficient of variation / ml	Calculated concentration /M
1.0	Water extract	10.43±0.096	5.000	0.918	1.043
	Methanol extract	10.38±0.096	2.828	0.923	1.038
	Acidified methanol extract	10.50±0.183	4.333	1.739	1.050
	Mixed indicator	10.18±0.096	n/a	0.941	1.018
0.5	Water extract	10.40±0.082	3.656	0.785	0.520
	Methanol extract	10.40±0.082	7.000	0.785	0.520
	Acidified methanol extract	10.58±0.096	12.124	0.905	0.529
	Mixed indicator	10.23±0.126	n/a	1.231	0.511
0.1	Water extract	10.35±0.058	4.899	0.558	0.1035
	Methanol extract	10.28±0.096	2.611	0.932	0.1028
	Acidified methanol extract	10.33±0.096	2.782	0.927	0.1033
	Mixed indicator	10.15±0.058	n/a	0.569	0.1015

SD: Standard deviation, t_c: critical value of t, n/a: not available.

Table 6: Average titres of titration of 0.1 M HCl with 0.1 M NaOH using anthocyanin extract from flowers *Hippeastrum hybridum* kept at room temperature through a period of 90 days.

Period in days		0	1	3	5	10	21	30	60	90
Average titre value /ml	OS	10.000	10.000	9.975	10.000	9.988	9.975	10.000	9.975	10.000
	DF	10.000	9.975	9.938	10.000	10.000	10.000	9.975	9.975	9.975
	PI	10.000	10.000	9.975	10.025	9.975	10.000	10.000	10.050	10.000
Calculated concentration M	OS	0.1000	0.1000	0.0998	0.1000	0.0999	0.0998	0.1000	0.0998	0.1000
	DF	0.1000	0.0998	0.0994	0.1000	0.1000	0.1000	0.0998	0.0998	0.0998
	PI	0.1000	0.1000	0.0998	0.1003	0.0998	0.1000	0.1000	0.1005	0.1000

OS: *Hippeastrum hybridum* flowers extract kept on open shelf,
DF: *Hippeastrum hybridum* flowers extract kept in deep freezer,
PI: Phenolphthalein Indicator.

DISCUSSION

In using anthocyanins of *Hippeastrum hybridum* to show endpoints in the titration of aqueous HCl versus aqueous NaOH, all the endpoints for all concentrations were very sharp and the results were accurate and precise. This is as the result of pH changes that take place at the endpoints. When a strong acid is titrated against a strong base, the equivalence point occurs at pH 7. However a sharp change in pH of the resultant solution ranging between pH 4 and pH 10 for reagents with a concentration of at least 0.1 M takes place (Harris, 2010). This pH change corresponds to the change of the anthocyanins from the flavylium ion form to the quinoidal form of which the two forms have different colors (Bondre et al., 2012).

In the titration of aqueous acetic acid versus aqueous NaOH, the anthocyanins of *Hippeastrum hybridum* showed accurate endpoint at all concentrations. In the titration of acetic acid with the sodium hydroxide, the pH of the resultant solution is above 7 (Harris, 2010). This pH of the resultant solution formed at the end point corresponds to the pH at which the anthocyanins have a different color from that in the acidic media.

In the titration of acetic acid against ammonia solution, the pH of the resultant solution formed at the endpoint is about pH 7.0 (Harris, 2010). This is the same pH about which the anthocyanins change from the flavylium cation to the quinoidal base hence showing color changes from red tones to blue tones. It is this reason that the anthocyanins in the methanol and acidified methanol extracts were able to show accurate endpoints similar to those obtained using mixed indicator when the concentration of the acid and base was 0.1 M. However, the titration of weak acid versus weak base is much affected by the concentration of the reagents. The effect of concentration on the accuracy of results in the titration of acetic acid versus ammonia comes from the instability of the ammonia solution where by dilute solutions of ammonia are more stable than concentrated solutions (Hales and Drewes, 1967). The molarity of the concentrated ammonium solutions keeps fluctuating which reduces the expected amount of base particles in the solution.

The anthocyanins of *Hippeastrum hybridum* were not suitable indicators in the titration of aqueous HCl against ammonia solution. In the titration of a strong acid versus a weak base, the pH of the resultant solution formed at the endpoint is below pH 7 (Harris, 2010). At such a pH, the anthocyanins still exist as flavylium cations, with colors in red tones. As the result, the anthocyanins would show a substantial color change after exceeding the endpoint which explains why they were predicting higher concentrations than the actual ones.

Conclusion

In using anthocyanins from *Hippeastrum hybridum,* to show endpoints in acid-base titrations, the sharpness of the endpoint, accuracy and precision of results are highly dependent on the type of acid-base combination (whether strong or weak) and less dependent on the concentration of reagents or the type of solvent used in the extraction. On average, the anthocyanins from *Hippeastrum hybridum* could be used in the titration of strong acid versus strong base, weak acid versus strong base and weak acid versus weak base. However, they did not work well in titration of strong acid versus weak base. The anthocyanins from *Hippeastrum hybridum* had a good shelf life of 90 days when kept at room temperature. The extracts can thus be used in any place including those that have no access to refrigeration facilities since they can be kept at room temperature for some time before they undergo degradation.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

All authors contributed to the design and writing of the proposal, obtaining results and in writing this manuscript.

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