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Full Length Research Paper

Use of anthocyanin extracted from natural plant materials to develop a pH test kit for measuring effluent from animal farms

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The aim of this work was to study the optimal conditions for anthocyanin extraction from natural plant materials in order to develop a pH test kit. The plant materials used were butterfly pea flower (BPF), roselle red flower (RRF) and dragon fruit peel (DFP). The solvents used in this study were distilled water, 1% HCl/95% ethanol (HE), 0.1 N acetic acid, 0.5% vinegar and 20% white liquor. A plant material-to-solvent ratio of 5: 50 (w/v) and an extraction time of 2 h were employed. The result shows that BPF could produce the highest average yield of anthocyanins, and that HE was the optimal solvent. Anthocyanins from the BPF+HE extract yielded the best properties for buffers in various pH, produced easily classifiable pH ranges, and were the most suitable to develop a pH test kit. In conclusion, the pH test kit developed by this method can be used for effluent measurement and will provide results similar in term of accuracy to those produced by a commercial pH test kit and a field pH meter.

Key words: Effluent, extraction of anthocyanin, pH test kit, water-soluble colorant.

INTRODUCTION

With the world human population estimated to increase from 5.40 billion in the 1990s to 8.50 billion by 2025, an increase in food production of 60 to 70% will become necessary to meet world food demands and minimize malnutrition (Power and Dick, 2000). Faced with a continuous decline in useful land for crop production, increased demands for animal products are likely to be met through more intensive agricultural production systems (Suppadit, 2009). In recent years, animal industries in Thailand have developed rapidly (Suppadit et al., 2006). The large-scale intensive farming of animals satisfies the demands of people for animal products; however, these farms result in large volumes of effluent in small regions, which creates serious pollution in the nearby environment (Suppadit et al., 2005a). According to one investigation, most intensive animal farms lack efficient effluent treatment methods and integrated utilization facilities (Suppadit, 2009). The animal farm effluent contains high concentrations of organic matters, suspended solids and NH_3-N (Suppadit et al., 2005b).

Optimal control of the effluent requires knowledge of its indicator characteristics, such as pH. A relatively high or low pH level in effluent should be of concern, as it may impact the local aquaculture and natural environment; the 6.5 to 9.0 pH range is usually suggested for fish culture (Lopes et al., 2001). Extreme pH values can lead to high

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Abbreviations: HE, Ethanol; **BPF,** butterfly pea flower; **RRF,** roselle red flower; **DFP,** dragon fruit peel; **DW,** distilled water.

mortality, while low pH reduces fish growth and reproduction (Boyd, 1998; Zweig et al., 1999). Therefore, accurate pH measurements are important to determine the state of raw effluent, to control the treatment steps used in neutralization of the effluent prior to discharge, and to monitor the quality of the final discharge in order to meet requirements for effluent standards (pH 5.0 to 9.0) (Pollution Control Department, 2000). The pH measurements vary according to the methods and instruments used (pH meter, pH paper and pH test kit). Extracting anthocyanins from natural plant materials to develop a pH test kit is one of the instruments that can be used to measure the pH of effluent.

Anthocyanins (from the Greek *anthos* = flower and *kianos* = blue) are the most important pigments of the vascular plants; they are harmless and can be easily incorporated into aqueous media (Choia et al., 2007; Hosseinian and Beta, 2007; Hosseinian et al., 2008), which makes them interesting for use as natural water-soluble colorants (Pazmino-Duran et al., 2001; Gonzalez-Mendoza et al., 2010). These pigments are responsible for the shiny orange, pink, red, violet and blue colors in the flowers and fruits of some plants (Lu et al., 2006; Castaneda-Ovando et al., 2009). Anthocyanins can be found in different chemical forms depending on the pH of the solution (Fleschhut et al., 2006; Guo et al., 2008; Sangkitikomol et al., 2010).

The objectives of this study were to determine: 1) the optimal conditions for extracting anthocyanins from natural plant materials, the kinds of solvents to use and the precision of anthocyanins in buffer solutions at different pH, 2) the stability of anthocyanin extracts during storage at various times and various temperatures and 3) the stability and accuracy of anthocyanin extracts (pH test kit) during testing with animal farm effluent, compared to the results from a commercial grade field pH meter and pH test kit.

MATERIALS AND METHODS

Experiment 1

Experiment 1 was designed as a 3 × 5 factorial arrangement with four replications (Chanthalukana, 1980). Two factors were used: 1) the type of natural plant materials, consisting of butterfly pea (*Clitoria ternatea* L.) flower (BPF), roselle red (*Hibiscus sabdariffa* L.) flower (RRF) and dragon fruit (*Hylocereus undatus* (Haw) Britt. & Rose.) peel (DFP), and 2) the type of solvents, consisting of distilled water (DW), 1% HCl/95% ethanol (HE), 0.1 N acetic acid (AA), 0.5% vinegar (V) and 20% white liquor (WL). The statistical analysis software (SAS) program version 6.12 (SAS Institute, 1996) was used to calculate the analysis of variance (ANOVA), and Duncan's new multiple range test was used to compare the experimental treatments.

The BPF, RRF and DFP were collected in June 2010 from a local market in Pathum Thani province, Thailand. The BPF was dried in a hot-air oven (Memmert, INB Model, Germany) at 50°C for 6 h, while the RRF and DFP were dried at 50°C for 48 h. Each of these materials was then ground using the sample mill (Krups Type 202, Germany) to pass through a 0.5 mm screen. The analyses were conducted on the ground samples. The data were expressed on dry weight basis.

All chemicals and solvents for extraction were of analytical grade. HCl, $C_2H_4O_2$, KCl, KH_2PO_4 and Na_2HPO_4 were purchased from Merck (Darmstadt, Germany). Ethanol (C_2H_6O), KHP and $Na_2B_4O_7$-$10H_2O$ were purchased from Ajax (Finechem, Australia). NaOH was purchased from Sigma-Aldrich (St. Louis, MO, USA). Vinegar (V) and white liquor (WL) were purchased from U&V Holding (Thailand) Co., Ltd. (Nonthaburi, Thailand).

For the experimental procedure, DW, HE (2.7 ml of 37% HCl + 97.3 ml DW (A) and 4.75 L of 100% ethanol + 0.25 L DW (B), and mix 50 ml of A + 4.95 L of B), AA (28.6 ml of 100% acetic acid + 4.97 L of DW), V (0.5 L of 5% vinegar + 4.5 L of DW) and WL (1.43 L of 70% white liquor + 3.57 L of DW) were prepared. The BPF, RRF and DFP (5 g) were then extracted with 50 ml of DW, HE, AA, V and WL, respectively, at room temperature and were shaken at 180 rpm for 2 h. Next, the crude extracts were filtered through a nylon filter and a filter paper (no. 1) and kept in an amber flask at 4°C. The total anthocyanin content was scanned on a UV-VIS Spectrophotometer (Cintra 10e, GBC, Australia) from 450 to 700 nm according to the Lees and Francis (1972) method. The UV-VIS spectral data were recorded and determined by the equation of Fuleki and Francis (1968).

The effect of pH on stability was also studied with 19 different buffers at the same temperatures. Buffer solutions at pH 1.0 to 13.0 were prepared as follows: pH 1.0 (50 ml of 0.2 M KCl and 134 ml of 0.2 M HCl); pH 2.0 (50 ml of 0.2 M KCl and 13 ml of 0.2 M HCl); pH 3.0 (100 ml of 0.1 M KHP and 44.6 ml of 0.1 M HCl); pH 4.0 (100 ml of 0.1 M KHP and 0.2 ml of 0.1 M HCl); pH 5.0 (100 ml of 0.1 M KHP and 45.2 ml of 0.1 M NaOH); pH 5.5 (100 ml of 0.1 M KHP and 73.2 ml of 0.1 M NaOH); pH 6.0 (100 ml of 0.1 M KH_2PO_4 and 11.2 ml of 0.1 M NaOH); pH 6.5 (100 ml of 0.1 M KH_2PO_4 and 27.8 ml of 0.1 M NaOH); pH 7.0 (100 ml of 0.1 M KH_2PO_4 and 58.2 ml of 0.1 M NaOH); pH 7.5 (100 ml of 0.1 M KH_2PO_4 and 82.2 ml of 0.1 M NaOH); pH 8.0 (100 ml of 0.025 M $Na_2B_4O_7$-$10H_2O$ and 41 ml of 0.1 M HCl); pH 8.5 (100 ml of 0.025 M $Na_2B_4O_7$-$10H_2O$ and 30.4 ml of 0.1 M HCl); pH 9.0 (100 ml of 0.025 M $Na_2B_4O_7$-$10H_2O$ and 9.2 ml of 0.1 M HCl); pH 9.5 (100 ml of 0.025 M $Na_2B_4O_7$-$10H_2O$ and 17.6 ml of 0.1 M NaOH); pH 10.0 (100 ml of 0.025 M $Na_2B_4O_7$-$10H_2O$ and 36.6 ml of 0.1 M NaOH); pH 10.5 (100 ml of 0.05 M Na_2HPO_4 and 8.2 ml of 0.1 M NaOH); pH 11.0 (100 ml of 0.05 M Na_2HPO_4 and 35.6 ml of 0.1 M NaOH); pH 12.0 (100 ml of 0.05 M Na_2HPO_4 and 53.8 ml of 0.1 M NaOH); pH 13.0 (50 ml of 0.2 M KCl and 132 ml of 0.1 M NaOH). 10 drops of each extract were added to each buffer solution and the color appearance was recorded.

The indicators used to choose the appropriate natural plant materials and solvents to study in experiment 2 were: 1) the ability to produce a distinguishing color in the buffer property of various pH (1.0 to 13.0) and the ease of classification of pH ranges consisting of strong acid (pH 1.0 to 3.0), weak acid (pH 4.0 to 6.0), effluent standard (pH 5.0 to 9.0) and base (pH 10 to 14); 2) the amount of anthocyanins; and 3) the availability of plant materials.

Experiment 2

The storage stability in terms of pH and the amount of selected anthocyanin extract were determined to develop an optimal pH test kit. Experiment 2 was designed as a 3 × 4 factorial arrangement with four replications (Chanthalukana, 1980). Two factors were used: 1) levels of storage temperature at 4, 25 and 30°C and 2) storage periods at one, two, three and four weeks. The SAS program version 6.12 (SAS Institute, 1996) was used to calculate the ANOVA, and Duncan's new multiple range test was used to compare treatments. The effect of temperature on the anthocyanin

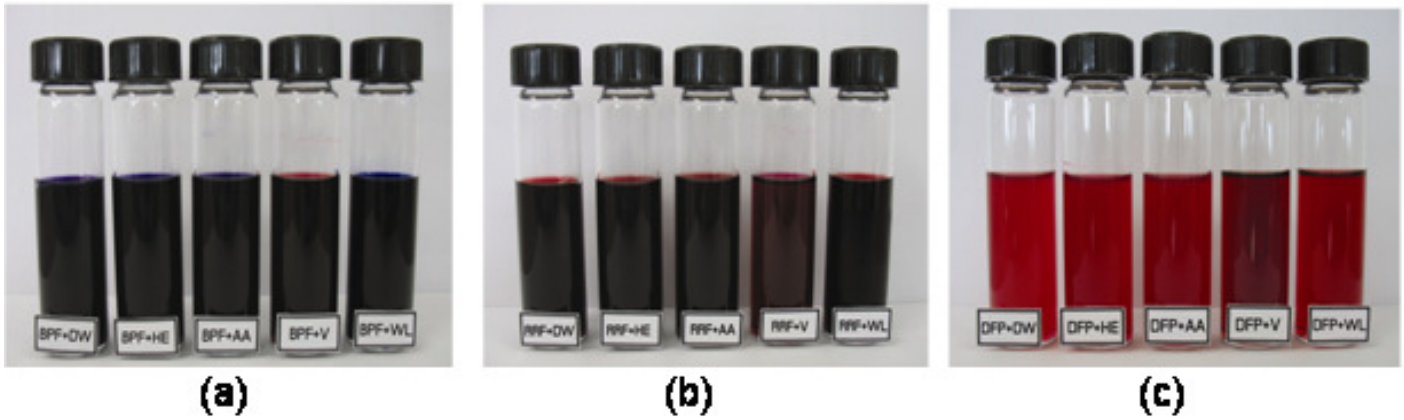

Figure 1. Anthocyanin extracts from (a) butterfly pea flower (BPF), (b) roselle red flower (RRF) and (c) dragon fruit peel (DFP).

extracts' stability after one and four weeks of storage were also tested with buffer solutions.

Experiment 3

To evaluate the accuracy of the developed pH test kit, experiment 3 was designed as a 3 × 5 factorial arrangement with four replications (Chanthalukana, 1980). Two factors were used: 1) the kind of instrument, consisting of the developed pH test kit, a commercial pH test kit and a field pH meter (PP-20, Sartorius, Germany) and 2) the source of effluent, consisting of a dairy cattle farm, beef cattle farm, laying quail farm, laying chicken farm and swine farm. All of the farms were located in the central zone of Thailand. The effluents were collected in the field using a grab sampling method and were tested with pH instruments. The obtained pH were recorded and the SAS program, version 6.12 (SAS Institute, 1996), was used to calculate the ANOVA, while Duncan's new multiple range test was used to compare treatments.

RESULTS AND DISCUSSION

Experiment 1

The pH of the DW, HE, AA, V and WL solvents were 6, 0.39, 2.66, 2.62 and 4.05, respectively. UV/VIS spectrophotometry was used to determine the absorption of UV/VIS light by a sample. Figures 1a to c shows the anthocyanin extracts from natural plant materials. The BPF yielded the highest amount of anthocyanins at 541 mg/100 g dry weight followed by RRF (280 mg/100 g) and DEP (172 mg/100 g), respectively ($P<0.05$) (Figure 2). For solvents, the DW used for the extraction yielded the highest amount of anthocyanins (394 mg/100 g dry weight) followed by WL (388 mg/100 g), HE (303 mg/100 g), AA (288 mg/100 g) and V (282 mg/100 g) ($P<0.05$) (Figure 2). The solvents had different material extraction molecule polarity, and the DW had a stronger polarity than other solvents (Jamikorn, 1996). Therefore, a hydrogen bond could occur between the solvent and the anthocyanins having the molecular structure of oxonium salt. It could be seen that the DW could extract anthocyanins more effectively than the other solvents.

Anthocyanins can well dissolve in a hydroxyl solvent, but cannot be dissolved in non-hydroxyl solvents such as ether, acetone and benzene (Castaneda-Ovando et al., 2009). Anthocyanins produce different colors depending on the pH of the solution. At pH 1.0, the flavylium cation (red) was the predominant species and contributed to violet and red. At pH between 2.0 and 4.0, the quinoidal blue species were predominant. At pH between 5.0 and 6.0, only two colorless species can be observed, which are a carbinol pseudobase and a chalcone, respectively. At pH higher than 7.0, the anthocyanins degrade depended on their substituent groups (Castaneda-Ovando et al., 2009).

Moreover, when the BPF+HE extract was tested with buffer solutions at pH 1.0 to 13.0, the colors appearing in solutions at pH 1.0 to 3.0 (strong acid), 4.0 to 6.0 (weak acid), 6.5 to 8.0 (effluent standard) and 8.5 to 13.0 (base) were red, violet up to blue, green and greenish yellow, respectively (Figure 3b). When the BPE+DW extract (Figure 3a), BPF+WL extract (Figure 3c), BPF+V extract (Figure 3d) and BPF+WL extract (Figure 3e) were tested with buffer solutions at pH 1.0 to 13.0, the colors appearing in solutions at pH 1.0 to 2.0 were all red, and at pH 3.0 to 13.0 the colors ranged from violet to yellow. The colors could not be separated by the pH range based on strong acid, weak acid, effluent standard, and base as they could with the BPF+HE extract. Furthermore, the RRF+HE extract was tested with buffer solutions at pH ranging from 1.0 to 13.0. The colors appearing insolutions at pH 1.0 to 5.0 (acid), 5.5 to 8.5 (effluent standard) and 9.0 to 13.0 (base) were red, brown and yellowish brown (Figure 4b), respectively. The RRF+DW extract (Figure 4a), RRF+AA extract (Figure 4c), RRF+V extract (Figure 4d) and RRF+WL extract (Figure 4e) were tested with buffer solutions at pH 1.0 to 13.0. At pH in the

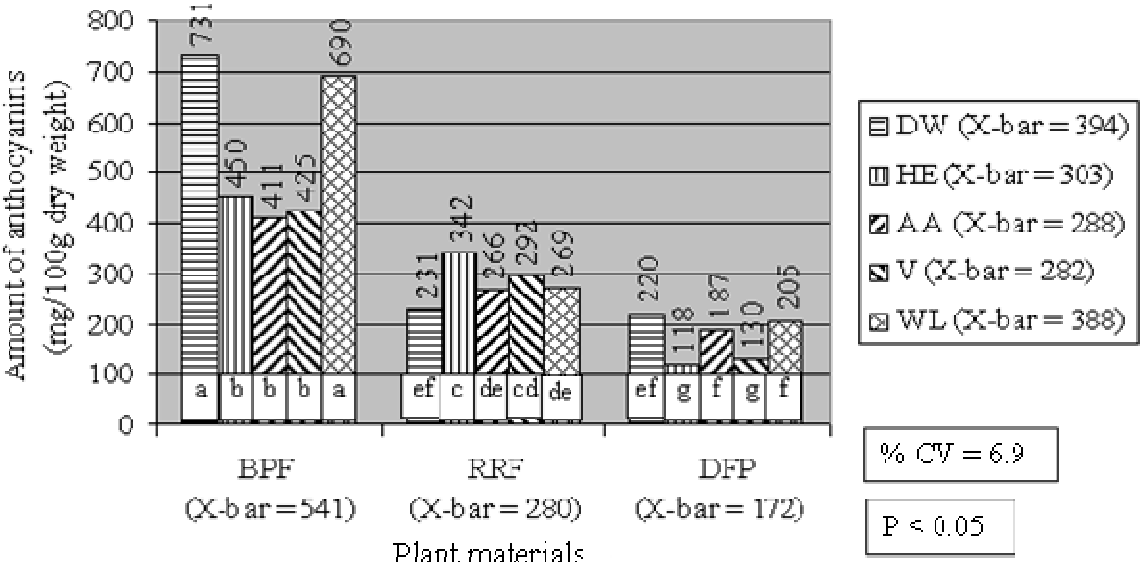

Figure 2. Amount of anthocyanins from BPF, RRF and DFP extracted by DW, HE, AA, V and WL solvents. BPF, butterfly pea flower; RRF, roselle red flower; DFP, dragon fruit peel.

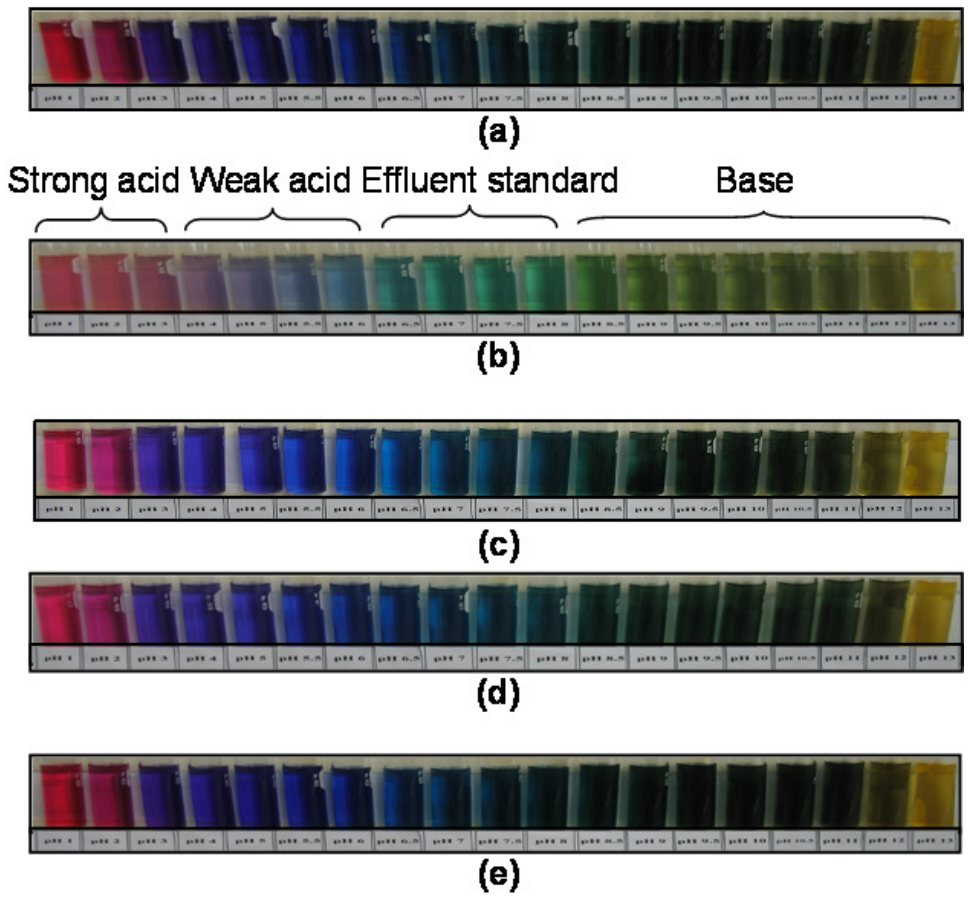

Figure 3. BPF+DW (a), BPF+HE (b), BPF+AA (c), BPF+V (d) and BPF+WL (e) extracts tested with buffer solutions. BPF, butterfly pea flower; HE, ethanol; AA, acetic acid; V, vinegar; WL, white liquor.

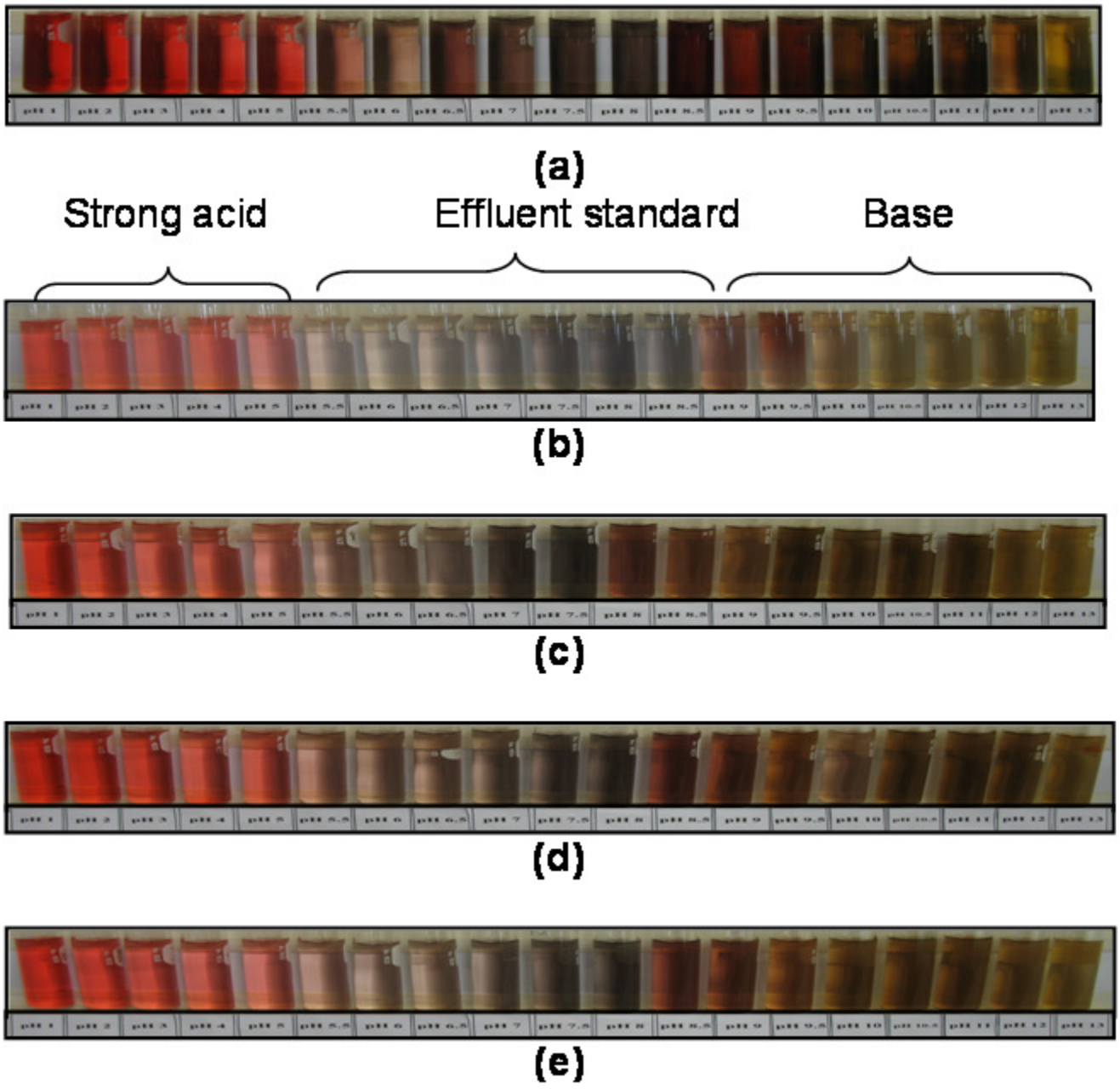

Figure 4. RRF+DW (a), RRF+HE (b), RRF+AA (c), RRF+V (d) and RRF+WL (e) extracts tested with buffer solutions. RRF, Roselle red flower; HE, ethanol; AA, acetic acid; V, vinegar; WL, white liquor.

1.0 to 5.0 range, the extracts produced red solutions, while at pH from 5.5 to 13; the extracts produced brown up to yellowish-brown solutions. The DFP+DW extract (Figure 5a), DFP+HE extract (Figure 5b), DFP+AA extract (Figure 5c), DFP+V extract (Figure 5d) and DFP+WL extract (Figure 5e) were tested with buffer solutions at pH 1.0 to 13.0. At pH 1.0 to 13.0, these extracts produced pink to yellow solutions. Moreover, strong acid, weak acid, effluent standard and base designations could not be sorted.

In conclusion, the most appropriate natural plant material and solvent to develop a pH test kit for measuring pH effluent were BPF and HE (BPF+HE extract) (pH 0.39) because the anthocyanins' stability in extract was influenced by the strong acid solvent (Castaneda-Ovando et al., 2009). Although the DW (pH 6.0) was the solvent that extracted the most anthocyanins, it could not produce clearly definable colors

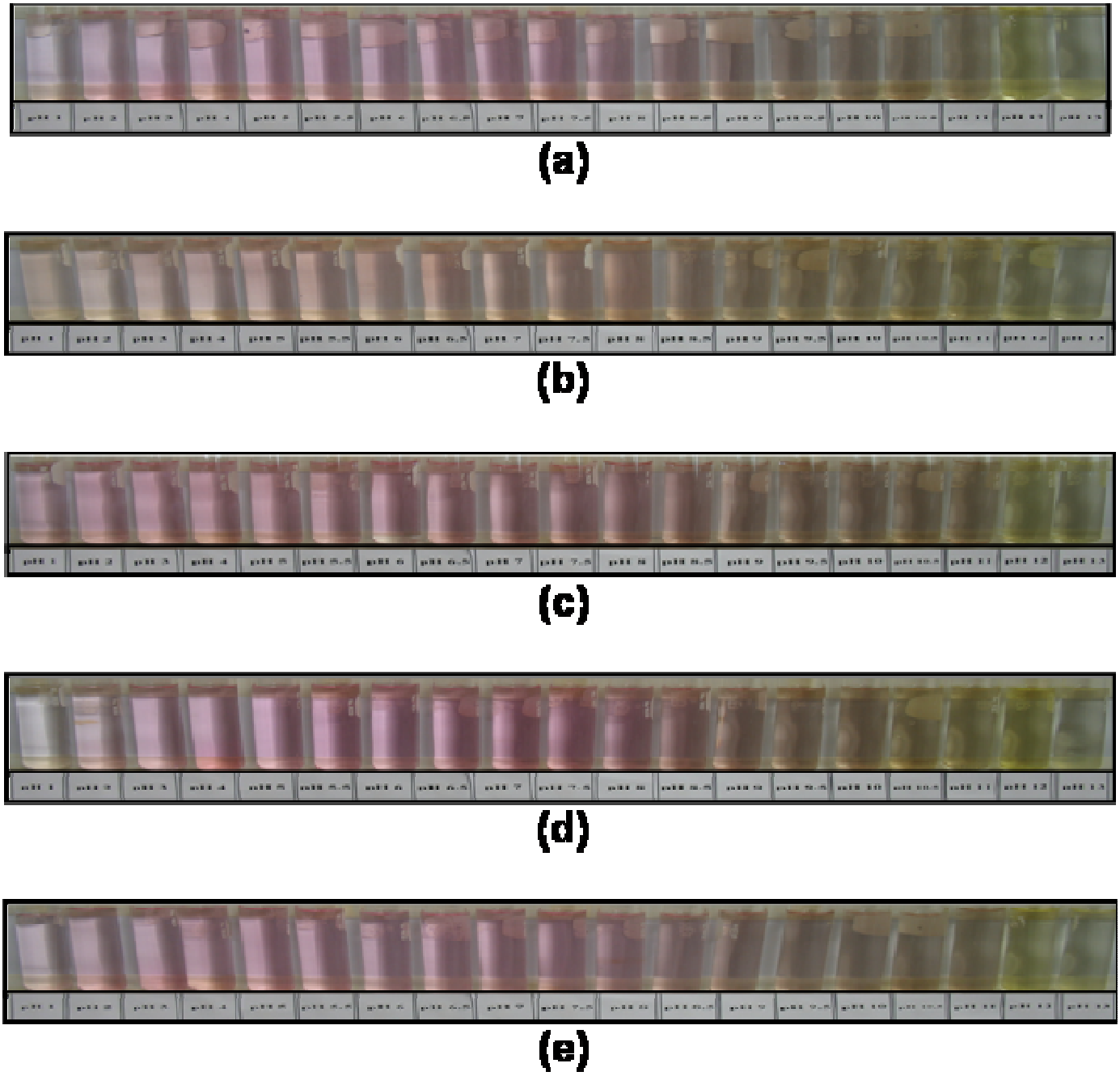

Figure 5. DFP+DW (a), DFP+HE (b), DFP+AA (c), DFP+V (d) and DFP+WL (e) extracts tested with buffer solutions. DFP, dragon fruit peel; HE, ethanol; AA, acetic acid; V, vinegar; WL, white liquor.

when tested with buffer solutions. The anthocyanins' stability in the extract decreases as the pH increases orin neutral and base media (Fleschhut et al., 2006).

Experiment 2

Based on the results of experiment 1, the BPE + HE extract was selected as the most appropriate for the development of a pH test kit. To further analyze the temperature effect on pH change, the BPF+HE extract was stored at 4, 25 and 30°C for four weeks. It was found that there was no significant difference in the pH of the BPE+HE extract stored at 4 (pH 1.34) and 25°C (pH 1.34) ($P>0.05$). However, there was a significant difference when stored at 30°C (pH 1.38) ($P<0.05$). A

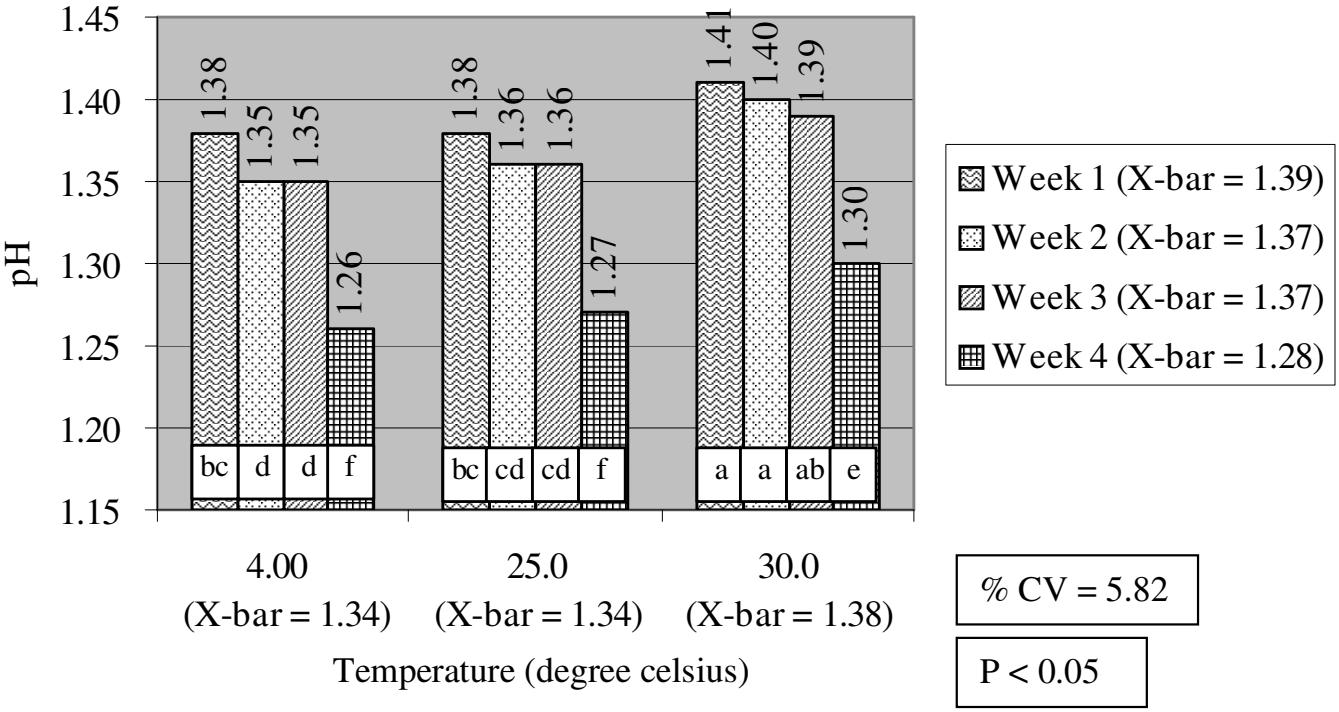

Figure 6. The pH of anthocyanin extracts stored at 4, 25 and 30°C for four weeks.

higher temperature could make the pH of the extract higher than that found in an extract stored at a low temperature. This effect might be because the temperature also found that the pH value decreased when the BPE + HE extract was stored for a long time (Figure 6). More also, with respect to the effect of temperature on the amount of anthocyanins in the BPF+HE extract at one to four weeks (4, 25 and 30°C), the storage of the BPE+HE extract at a higher temperature and longer period of time decreased the amount of anthocyanins ($P<0.05$). This property was due to the fact that a high temperature and long period of time affected the anthocyanins' structure, and two colorless species could be observed; a carbinol pseudobase and a chalcone (Kirca et al., 2007) (Figure 7). These results agree with those obtained by previous studies reporting the degradation of anthocyanins from temperature and storage time (Kirca and Cemeroglu, 2003; Rubinskiene et al., 2005; Bordignon-Luiz et al., 2007; Hosseinian et al., 2008; Lo Scalzo et al., 2008).

In addition, the pH test kit produced from the BPF+HE extract was tested with buffer solutions at pH 1 to 13 before and after storing at 4, 25 and 30°C for four weeks. The BPF+HE extract stored at 4 and 25°C for four weeks produced color in solutions that could be sorted into strong acid, weak acid, effluent standard and base, as they had before storing. However, after storing at 4°C and testing with buffer solution, the color appearing in solution was clearer. Moreover, after storing at 30°C for affected the pH of the extract in the acid condition; therefore, the pH of the extract could be changed depending on temperature (Miguel et al., 2004). It was for weeks and testing with buffer solutions, the appearing colors could not be separated by the pH ranges of the buffer solution (Figures 8a to d) because the temperature affected the anthocyanins' structure, and colorless species were observed (carbinol pseudobase and chalcone) (Kirca et al., 2007). In conclusion, to minimize extract degradation, the BPF + HE extract should be cooled, possibly to refrigeration temperatures (~4°C), as soon as produced.

Experiment 3

When the BPF+HE extract developed as a pH test kit, was tested with five sources of animal farm effluent samples and compared with color bars on Figure 3b for the evaluation of pH, it was found that the color occurred in the effluent standard of all sources (pH 5.0 to 9.0) (Figure 9). Comparing the obtained pH values, the developed pH test kit measured the pH of the effluents with results similar to those obtained with a commercial pH test kit and field pH meter, with no significant difference ($P>0.05$) (Figure 10). Therefore, it can be concluded that the developed pH test kit can be used to measure the pH values by vision in actual field conditions.

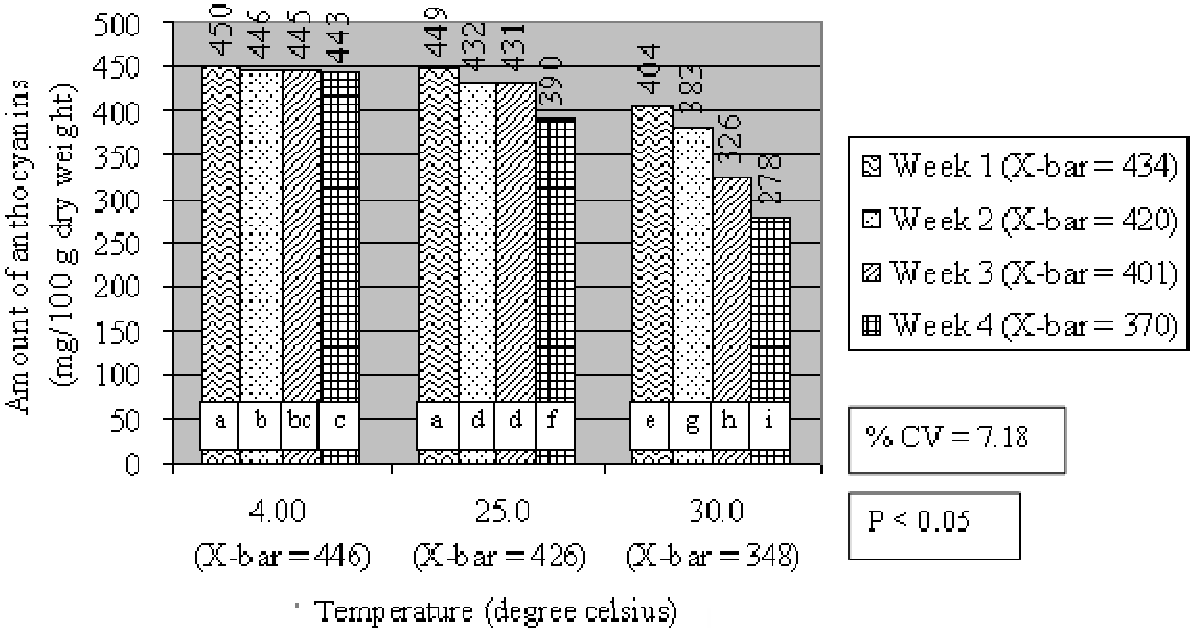

Figure 7. Amount of anthocyanin extracts stored at 4, 25 and 30°C for four weeks.

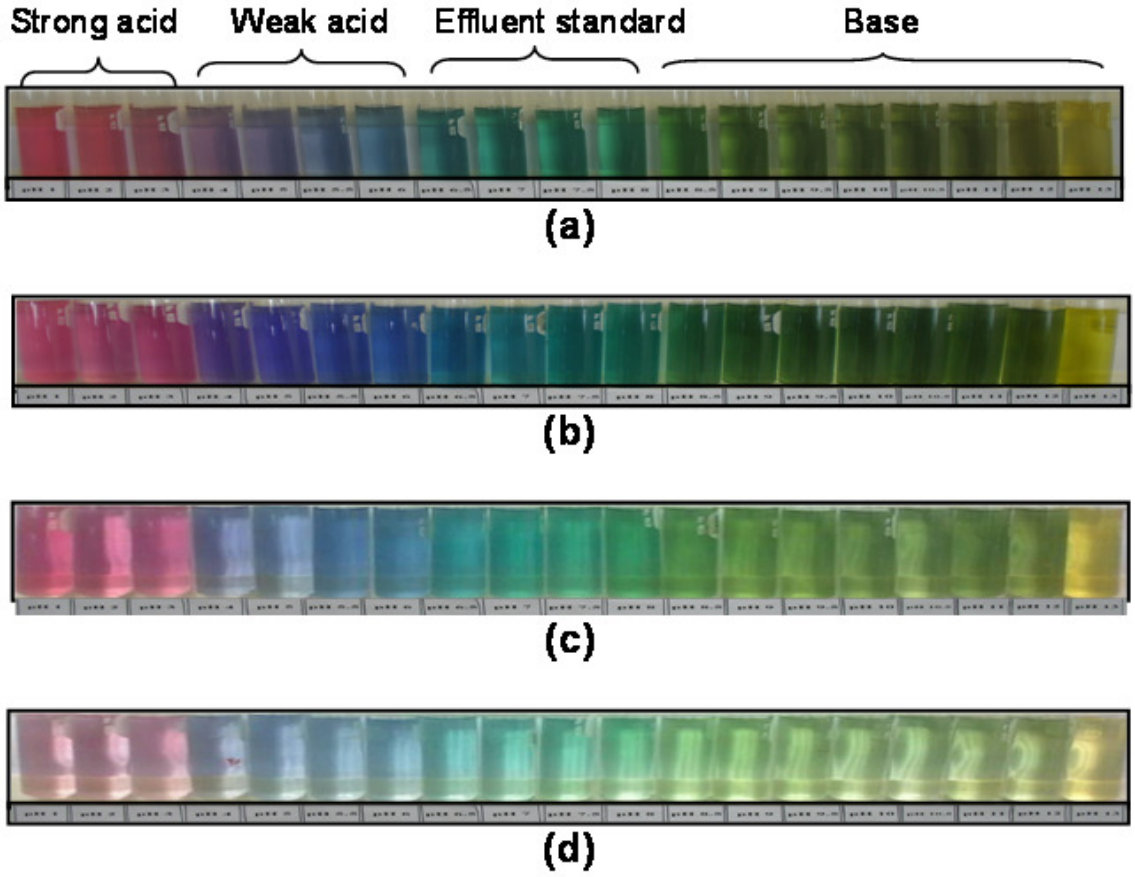

Figure 8. (a) Before storage and (b) after storage at 4, (c) 25.0 and (d) 30.0°C for four weeks.

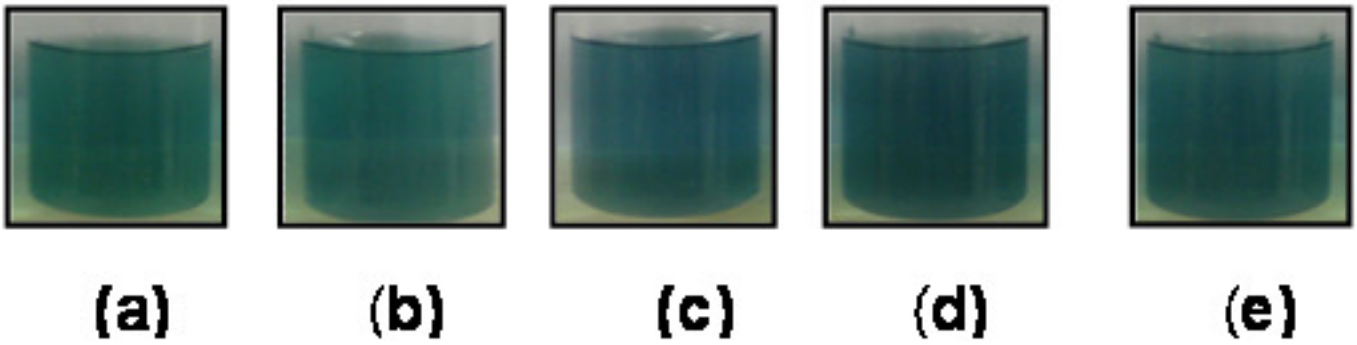

Figure 9. (a) Measuring pH effluent of dairy cattle farm, (b) beef cattle farm, (c) laying quail farm, (d) laying chicken farm and (e) swine farm by comparing with the color bars on Figure 3b.

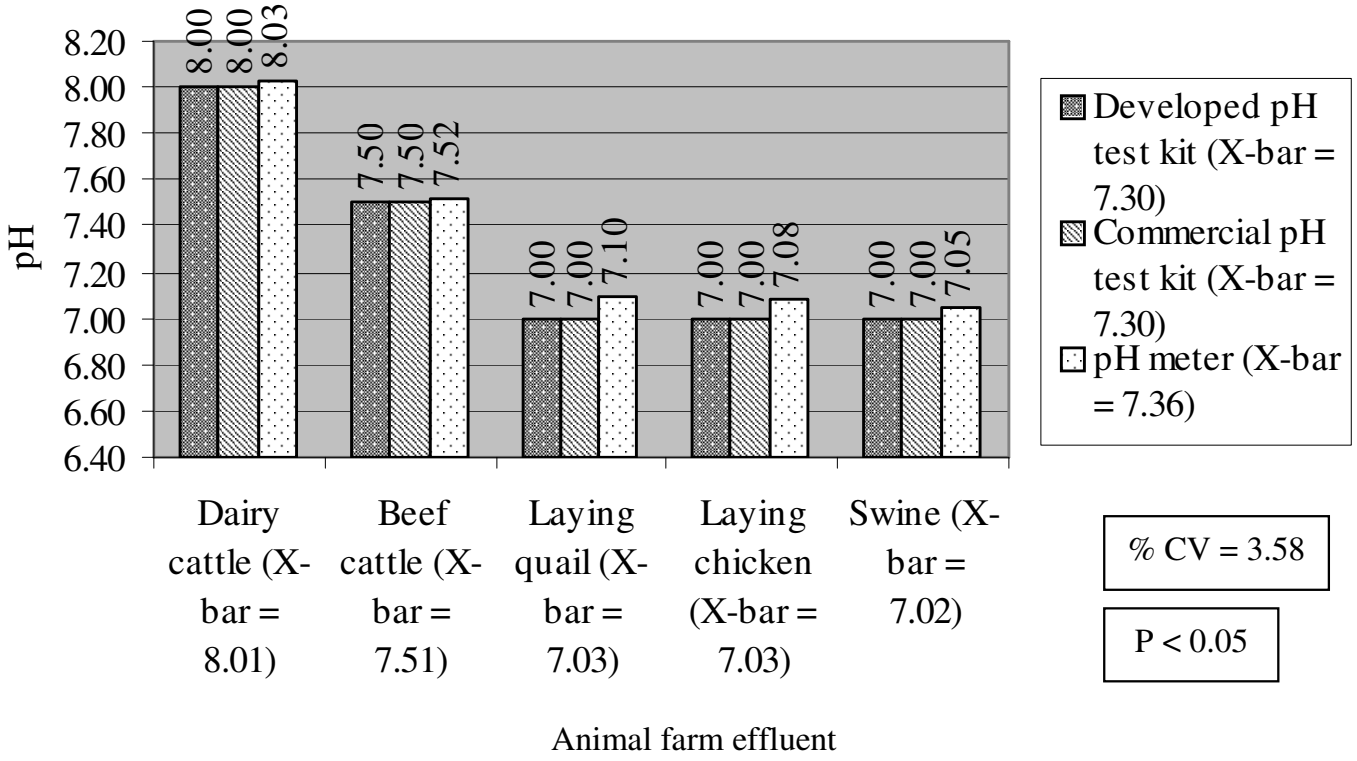

Figure 10. Comparison of pH in animal farm effluents among developed pH test kit, commercial pH test kit and pH meter.

Conclusion

Anthocyanins could be extracted by using 5 g of BPF and 50 ml of HE solvent. Briefly, the BPF was dried at 50°C for 6 h and ground; it was subsequently shaken in a shaker at 180 rpm for 2 h and sifted through a nylon filter and a filter paper (no.1). The obtained BPF+HE extract volume was 35 ml. The extract was kept in an amber bottle at 4°C. The pH test kit consisted of the following: 1) pH testing liquid (BPF+HE extract), 2) color bars standard for the evaluation of pH (Figure 11), adapted from Figure 3b, and 3) 15 ml test tube. The testing process consists of the following: 1) put a 10 ml effluent sample into the test tube, 2) add 10 drops of testing liquid; 3) cover the tube and shaking for 2 or 3 min, and 4) compare the color to the color bars standard (Figure 11) to observe the pH. The pH test kit developed can measure the pH value of animal farm effluent in term of accuracy as well as any commercial pH test kit and field pH meter. Moreover, the outputs from this method hope to establish fundamental method and contribute to optimized pH test kit efficiency in terms of production cost and self-interest in developing

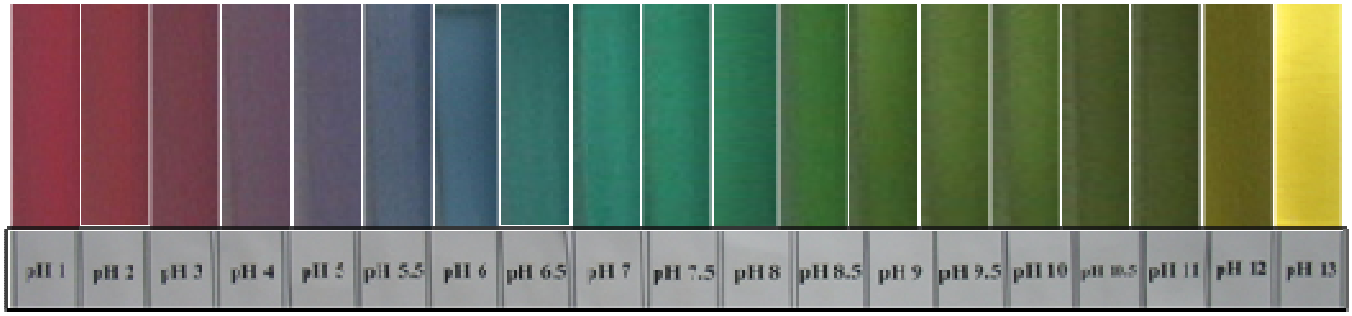

Figure 11. Developed color-bar standard for the evaluation of pH.

country.

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