The Elucidation of the Saffron Petal based Biosensor Mechanism in the Identification of Rotten Fruits by HPLC

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Abstract: Sensors and biosensors are used in many identification techniques. The construction and design of different biosensors is different according to the need and type of work, and the mechanism of action is different depending on the chemistry of the raw materials.
The saffron petals are used asthe basis of the plant biosensors for the detection of rotten fruits.Based on the color change from green to red, the fruits spoilage is detected.To elucidate the mechanism of the introduced biosensors, HPLC with UV detector and C18 column isused.UV-Vis spectrophotometers are also utilized to evaluate its efficiency.
Examination of the obtained spectra revealed that the effective color dye in saffron petals is anthocyanin, which is converted to flavonoids in acidic pH medium, and this conversion causes red color.
In the assessment of the reproduction of the prepared biosensor, it is found that the sensor made of saffron petals is completely amplifiable.

Keywords: Plant biosensor, saffron petals, reproduction, spectrophotometer, anthocyanin, flavonoid, HPLC.

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Introduction:

Biosensors come in an increasing array of forms with a rapid developmentrate. However there are extremely useful biosensors for some signals, most are still remained qualitative. Other qualities sought in biosensors are temporal and spatial resolution and, usually, an ability to use them without significantly perturbing the system (1).
Biosensor, which is used for the detection of particular analytes, is ananalytical device that works based on physicochemical reactions. Biosensors arepromising bio-devices, which can be used as substitutes for the traditional analyticalmethods, for rapid, easy, economical and reliable analysis(2).
Saffron petals can be used as a biosensor due to its color change in different environments. The suitability of this plant for identifying rotten fruits has already been confirmed and its results have been published by our research team (3).
In the present study, the cause of color change and also the reproductionof this biosensor are investigated.

Anthocyanins are phenolic water-soluble pigments. The pigments are in glycosylated forms(4).Anthocyanins is responsible for thered, purple, and bluecolorsin fruits and vegetables(5). Berries, currants, grapes, and some tropical fruits have high anthocyanins content. Red to purplish blue-colored leafy vegetables, grains, roots, and tubers are the edible vegetables that contain a high level of anthocyanins. Among the anthocyanin pigments, cyanidin-3-glucoside is the major anthocyanin found in most of the plants. The anthocyanin pigments have been traditionally used as a natural food colorant(6). The color and stability of these pigments are influenced by pH, light, and temperature(7). In acidic condition, anthocyanins appear as red but turn blue when the pH increases.

Anthocyanins are glycosylated polyhydroxyandpolymethoxyderivatives of 2-phenylbenzopyrylium (flavylium) salt, which are natural pigments widely distributed in nature (8).Owing to their specific pyrylium nucleus (C-ring), anthocyanins express a much richer chemical reactivity than the other flavonoid classes. For instance, anthocyanins are weak diacids, hard and soft electrophiles, nucleophiles, prone to developing -stacking interactions, and bind hard metal ions. They also display the usual chemical properties of polyphenols, such as electron donation and affinity for proteins(9).Flavonoids are a class of polyphenolic compounds that naturally occur in plants (10).

As can be seen in the figure 1, a simple structural change from anthocyanin to flavonoids occurs in an acidic medium. This structure belongs to the purple cabbage anthocyanins.

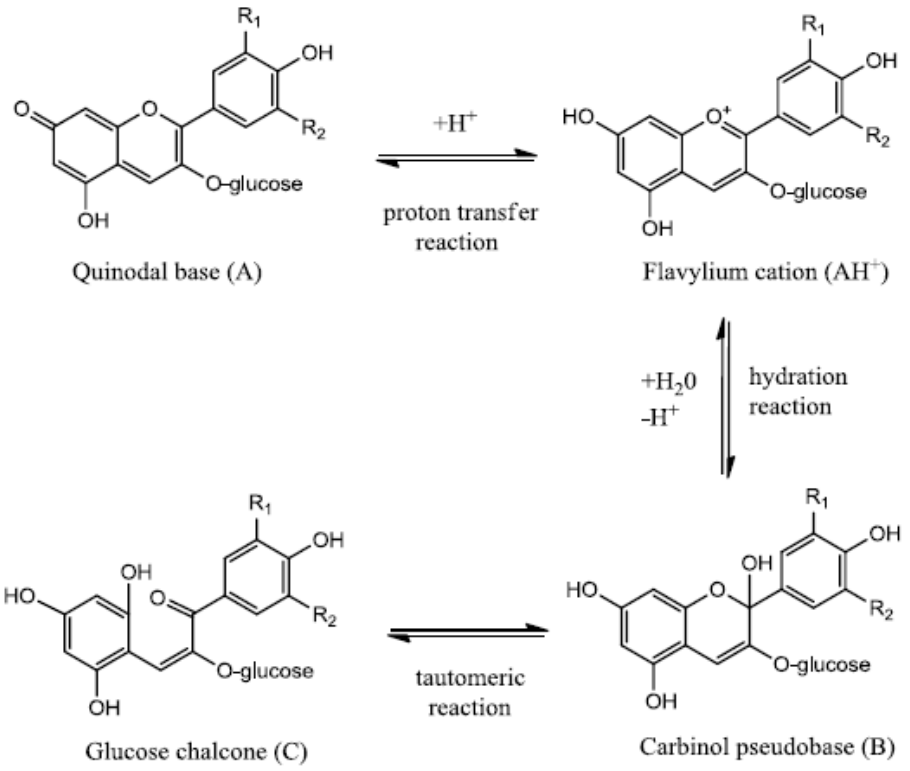

Figure 1. Conversion of anthocyanin to flavonoid in the range of acidic pH (13)

Chromatography has been largely applied in extraction, separation, and quantification of anthocyanins (11-12).

In this article, the effective substance in saffron petals is evaluated by HPLC. The change of color of this substance in acidic environment is also investigated.
Then, the reproduction of the biosensor made of saffron petals is measured and verified by three different spectrophotometers.

Materials and Methods:

To prepare the aqueous extract of saffron petals, first the petalscollected from the farms of Bojnourd city (North Khorasan province, Iran) were dried for 14 days in a dry environmentand under sunlight. 1 gr of dried saffron petals was added to 100 cc of distilled waterat 95 ° C. After ten minutes, the solution was filtered through a thin cloth. The calc paper, measuring 2 cm by 7cm , was then immersed in the solution for 12 hours at 75 ° C. After 12 hours, the biosensor was ready to use. Then the obtained aqueous extract was used for injection into the HPLC device(3).

Figure 2: A is saffron and saffron farm. B is Dried saffron petals. C is Aqueous extract of saffron petals D is biosensor

Figure 2A shows the saffron flowers grown in the saffron field. These color of the flower petals are changed to dark blue after harvesting. The blue petals were dried in a dark and clean environment (Figure 2B). The dried petals were thenput in double-distilled boiled water for ten minutes (2C).The aqueous extract of saffron petals was then fixed on tracing paper (3) and the desired biosensor was made (2 D).
As the blue fruits contain the anthocyanin, pure HPLC-gradeanthocyanin was injected into the device.The change of anthocyanin to red color in acidic medium according to the literature (9)is due to its conversion to flavonoids. To test it, pure flavonoid was preparedfromSigma (Germany) for injection in HPLC. These materials were injected into a HPLC (Perkin Elmer Series 200, USA)with UV detector and C18 column using anautosampler.
Methanol (HPLC Grade), acetonitrile (HPLC Grade), water (HPLC Grade),trifluoroacetic acid (HPLC Grade), formic acid (>99%), citric acid, potassium chloride, and sodium acetate were purchased from Merck (Pittsburgh, PA, USA).Ethanol (ACS/USP Grade, 200 proof) was purchased from Pharmco Products(Brookfield, CT, USA).

HPLC Conditions

A flow rate of 1.0 mL/min was used. The injection volume was 5 µL for eachsample. The column temperature was 35 ± 5°C. UV-Vis detection was at 520 nm. Asolvent gradient was utilized. For 3 minutes, 95% B (0.1% TFA) flowed through thecolumn. From 3 to 25 minutes, a

linear gradient went to 5% B and from 25 to 30 minutesthe column was re-equilibrated to 95% B(13)

Reproducibility means that the biosensor designed by several similar devices has the same answers or biosensor tests are performed by different people and close answers are obtained.For this purpose, experiments were performed with three spectroscopic devices with the Lebomed (Germany), Perkin Elmer (USA), and PG 80 Plus (England), double beam model.
After preparing the solutions and aqueous extract of saffron petals, the spectrum of the solution was taken and compared with the spectrum of pure anthocyanin and then the buffer was injected with pH=3 and the spectrum was taken again and compared with the spectrum of pure flavonoids.
The observational tests were performed randomly by 20 people to confirm the reproducibility of the prepared biosensor.

RESULTS
The HPLCspectra of pure anthocyanin and aqueous extract of saffron petals are shown in Figure 3.

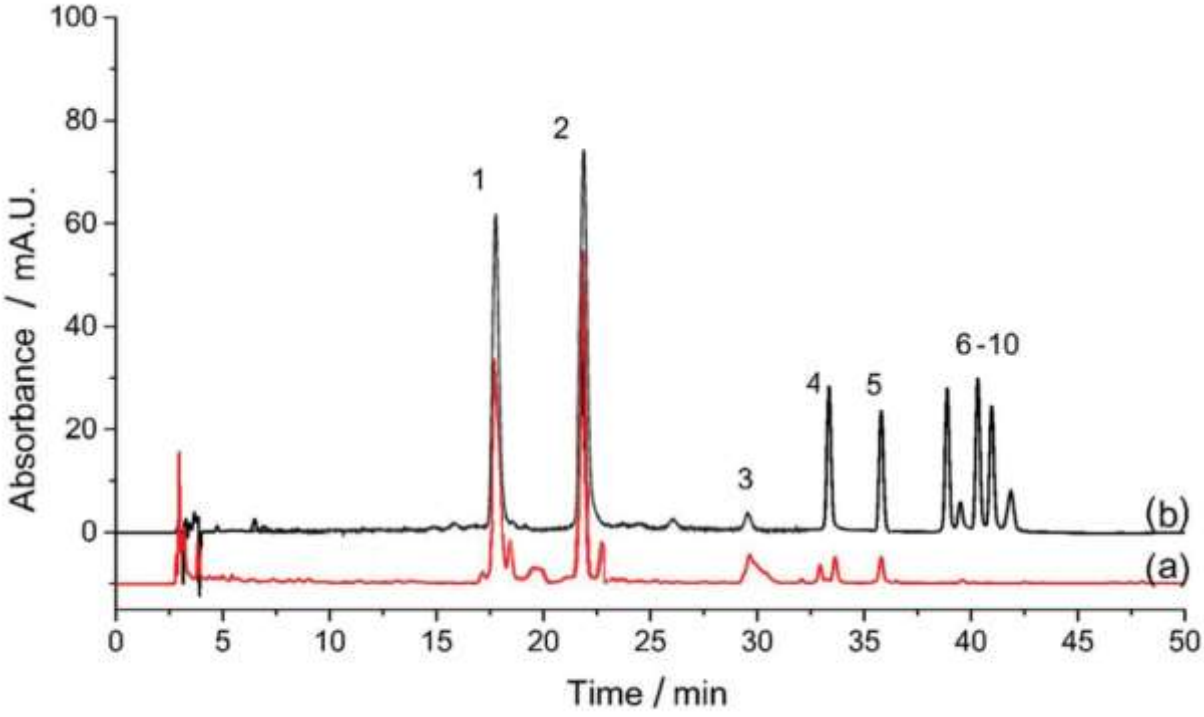

Figure3. (a) Pure anthocyanin spectrum (b) Saffron petal extract and with column C18

As shown in Figure 3; the pure anthocyanins (spectrum(a) have two clear peaks at 17 and 22 minutes. In spectrum(b), which is attributed to the saffron petal extract, these two index peaks can be seen at 17 and 22 minutes.
These two peaks clearly prove that the colorant in the saffron petals is due to the presence of anthocyanin compounds.

Then, saffron petal extract was injected with pH= 3. Anthocyanin is converted to flavonoids in acidic medium and has a red color (9). The collected spectrums of saffron petals in the presence of buffer and the pure flavonoids are compared (Fig. 4).

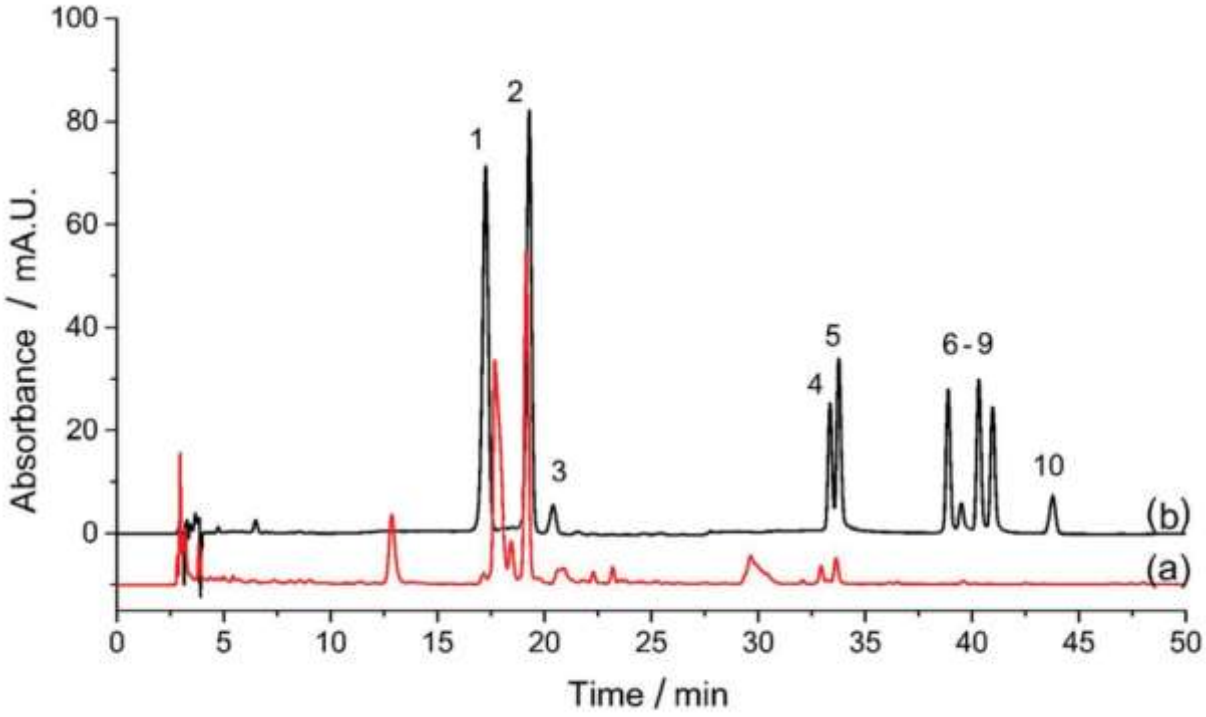

Figure4. Spectrum(a) is pure flavonoid (b) is saffron petal extract after adding buffer with acidic pH

Nathan Blain Stebbins, examined the NMR spectra and spectrophotometers of pigments in blue fruits such as blueberries, blue grapes, etc., and showed that the color of the extracts of these fruits changed in the acidic pH range of blue to red is due to the conversion of anthocyanins to flavonoids (Fig. 1)(12).

As we see in Figure 1, Nathan's report reinforces the possibility that the green to red color change of the saffron petal extract in the acidic pH range is the conversion of anthocyanins to flavonoids.

Therefore, the HPLC chromatogram of saffron petal extract was taken after adding buffer with pH= 3. The results can be seen in the Fig 4.

First a pure flavonoids and then the saffron petal extract containing buffer with acidic pH (pH=3)were injected into the device and the chromatograms were collected.

According to Figure 4, it can be seen that the red color of saffron petal extract after adding buffer with acidic pH, is due to the presence of flavonoids.

As shown in the spectrum, the pure flavonoids (spectrum (a)) have two distinct peaks at 16 and 18 minutes. In the spectrum of saffron petal extract containing acidic buffer (spectrum (b)), these two characteristic peaks are also observed, which the reason for the presence of flavonoids is.

It is proved by comparing the two spectra of HPLC taken from saffron petal extract before and after adding acid buffer; the color change is due to the conversion of anthocyanins to flavonoids in saffron petal extract.

The data of the spectra from HPLCindicated that the molecular structure of anthocyaninsfrom Saffron petals matched withcyanidin 3-*O*-rutinoside, (structures see Figure 3). Peak1 was identified as Cyanidin-3-(caffeoyl)-diglucoside-5-glucoside‹ peak 2 was identified as Cyanidin-3-(feruloyl)(feruloyl)-diglucoside-5-glucoside (14).
The data of the spectra from HPLC- UV indicated that the molecular structure of flavonoids from Saffron petals matched with flavonolPeak1(structures see Figure 4) was identified asCyanidin-3-(caffeoyl)-diglucoside-5-glucoside peak 2 was Petunidin-3-*O*-arabinoside peak 5 was identified as malvidin 3-*O*-(6 acetyl) galactoside.

Evaluation of reproducibility of saffron petal biosensor
The spectrums of saffron petal extract which are taken by three spectrophotometers Lebomed, Perkin Elmer, and PG80+are presented in the Figure 5.

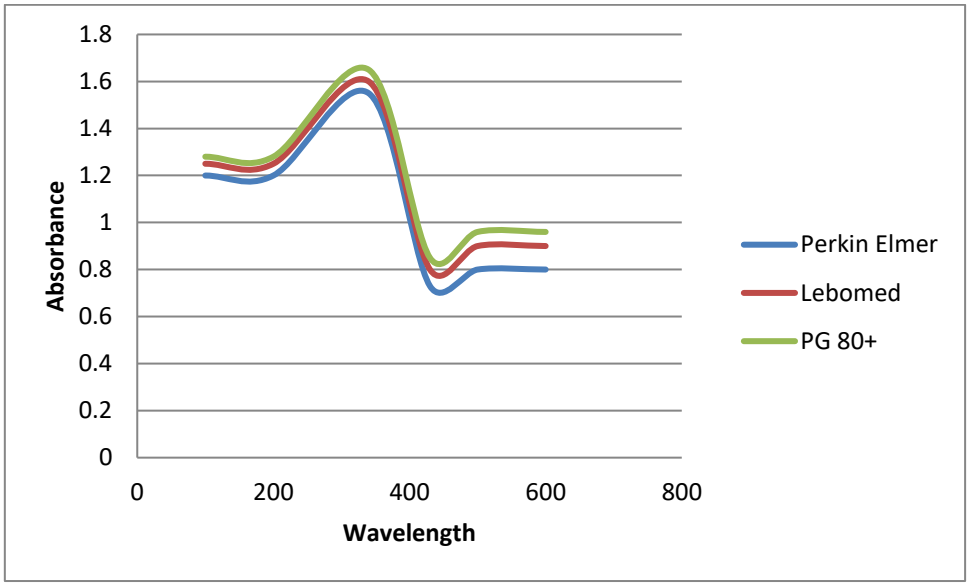

Figure 5: Spectrum of pure saffron petal extract by Lebomed, Perkin Elmer, and PG80+spectrophotometer

It shows that the spectrum obtained from the aqueous extract of saffron petals is the same in different devices.The slight difference in their absorption rate is related to the type of lamp, the light intensity, detector sensitivity, and scanning speed in different devices. But in general, the shape of the spectrum, peaks and valleys are identical at specific wavelengths, which is the same in all three devices, and the shape of the resulting spectrum is the same. Thus, thereproducibilityof saffron petal solution is well confirmed.

Biosensor spectrum in the vicinity of the buffer
Aqueous extracts of saffron petals wereevaluated in the presence of buffers with acidic pHs in the pH range 1 to 7, the results arepresented in the Tables 1,2, and 3 and Figures 8, 9, and 10.

Table 1 Wavelengths and adsorption of saffron petal extract at different pHs by Lebomeddevice

Wavelength(nm)	pH=1	pH=2	pH=3	pH=4	pH=5	pH=6	pH=7	Original
100	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
200	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
340	1.7	1.6	1.55	1.5	1.43	1.4	1.35	1.3
430	0.7	0.72	0.73	0.74	0.75	0.76	0.77	0.77
500	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
600	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8

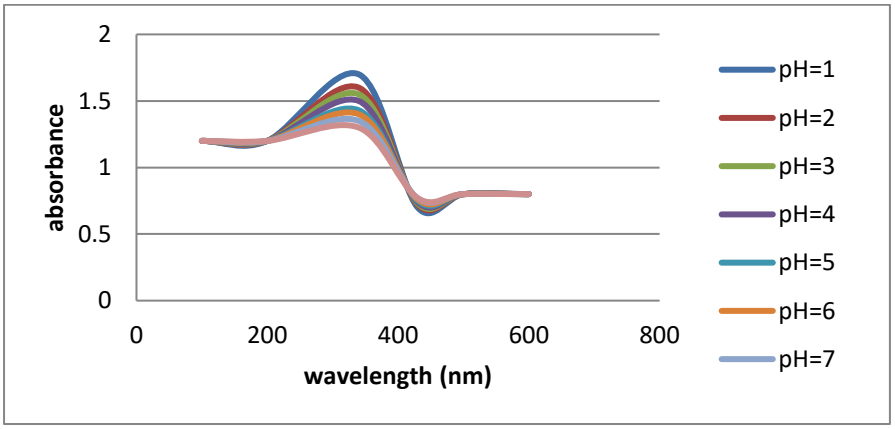

Figure 6: Spectrum of saffron petal extract exposed to pH buffers 1 to 7 by Lebomed device

Table 2: Wavelengths and absorption of saffron petal extract at different pHs by Perkin Elmer

Wavelength(nm)	pH=1	pH=2	pH=3	pH=4	pH=5	pH=6	pH=7	original
100	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
200	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
340	1.6	1.55	1.52	1.45	1.4	1.38	1.26	1.2
430	0.65	0.67	0.68	0.69	0.7	0.71	0.72	0.72
500	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
600	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7

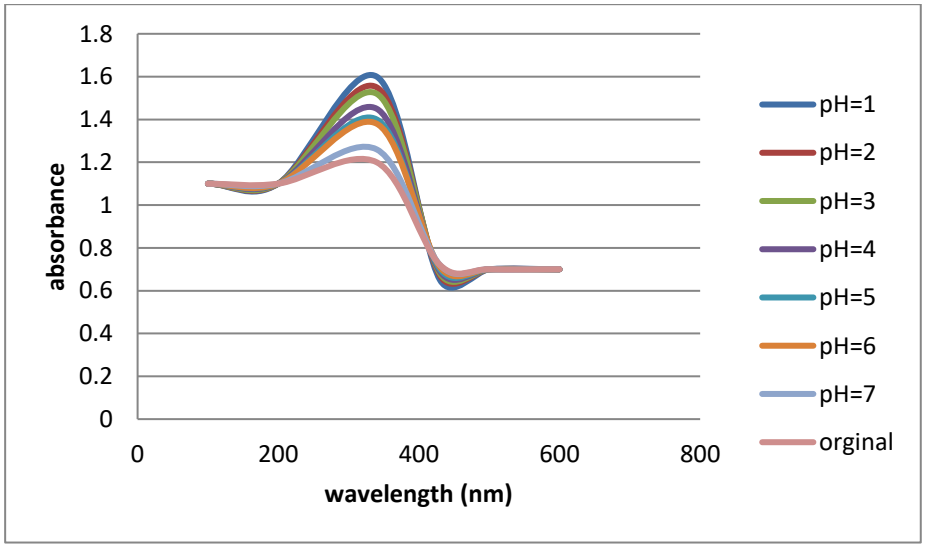

Figure 7: Spectrum of saffron petal extract exposed to pH 1 to 7 buffers by Perkin Elmer device

Table 3 :Wavelengths and uptake of saffron petal extract at different pHs by PG 80 Plus

Wavelength(nm)	pH=1	pH=2	pH=3	pH=4	pH=5	pH=6	pH=7	Original
100	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
200	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
340	1.82	1.71	1.66	1.6	1.54	1.51	1.46	1.4
430	0.79	0.81	0.82	0.83	0.85	0.87	0.89	0.89
500	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
600	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9

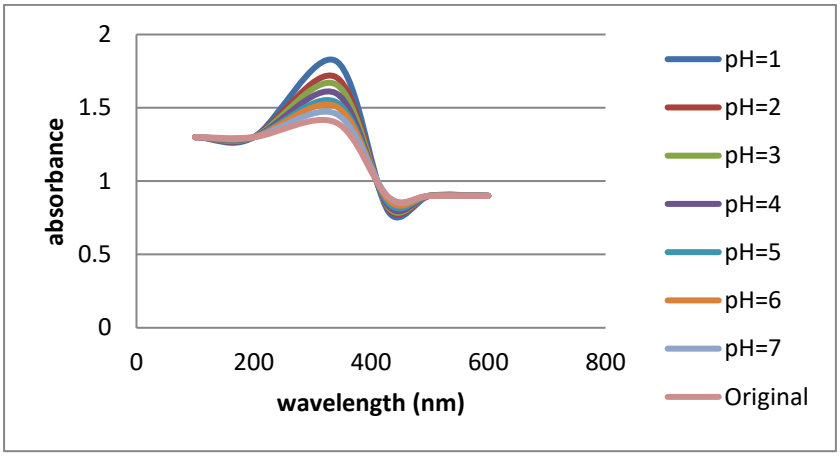

Figure 8: Spectrum of saffron petal extract exposed to pH buffers 1 to 7 by British PG 80 Plus

The results of saffron petal solution in the vicinity of acidic buffers shown in the Tables 1 to 3 and Figures 6to 8of demonstrate that these solutions have valleys and peaks atthe same points, which indicates that the test route is correct.

According to the Tables 1 to 3 and Figures 8 to 10, by injecting buffers with different pHs, clear changes in adsorption can be seen, with the highest absorption at pH = 1 and the lowest absorption at pH = 7, indicating an obvious response. This indicator indicates the acidic region and suitability for this detector.

The similarity of the spectrums of saffron petal solutions in different pHcollected by three different devices indicated that the biosensor of saffron petals is reproducible.

To evaluateanother aspect of reproducibility, it was necessary to examine human error. To do this, the preparedmembranes were randomly tested by 20 people. The color of the membrane in the normal state and in contact with the rotten fruit was asked them individually. Only in one case, which had color blindness, a different membrane color was reported. In the other 19 cases, all indicated correctly green and red colors. The results are shown in Table 4.

Table 4: Volunteers' responses in using the saffron petal biosensor

Membrane color observed in contact with rotten fruit	Normal membrane color observed	Gender	education	Individual age	Row
Red	Green	Female	bachelor's degree in industrial engineering	23	1
Red	Green	Man	Diploma Humanities	18	2
Red	Green	Female	Second elementary	8	3

Red	Green	Man	sixth elementary	63	4
Red	Green	Man	Preschool	6	5
Red	Green	Female	Fifth elementary	75	6
Brown	Brown	Man	MA	45 (Blind color)	7
Red	Green	Female	Diploma	25	8
Red	Green	Man	P.H.D	36	9
Red	Green	Man	P.H.D	38	10
Red	Green	Female	illiterate	4	11
Red	Green	Man	MA	43	12
Red	Green	Female	First High School	14	13
Red	Green	Female	Bachelor	27	14
Red	Green	Man	Diploma	54	15
Red	Green	Man	sixth elementary	67	16
Red	Green	Female	illiterate	70	17
Red	Green	Female	MA	33	18
Red	Green	Man	MA	21	19
Red	Green	Man	Fourth Elementary	10	20

What emerges from Table 4 and the comparison of the data and its results is that the built-in biosensor responds to all ages, levels of literacy, and gender appropriately.
The biosensoris easyto use, and has reproducibility. However, since this biosensor is based on color change, it is not useful for people with color blindness.

Conclusion

Based on the results obtained from the HPLCspectra, it was confirmed that the reason for the change in color of the aqueous extract of saffron petals from green to red in the presence of acidic medium isthe conversion of anthocyanins to flavonoids.
The reproduction of the saffron petals based biosensor in the detection of rotten fruits was proved using three double-beam spectrophotometers with different brands. The comparing the spectra confirmed that this biosensor is completely amplifiable. To investigate another aspect of the reproductionof this biosensor, 20 people of different situations participated in this experiment and the results of their answers strongly proved thereproduction of this plant biosensor.

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