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Separation and Determination of Some Organic Acids in Dry Calyces of Iraqi Hibiscus Sabdariffa Linn

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Abstract:

A new reversed phase- high performance liquid chromatographic (RP-HPLC) method with Ultraviolet-Visible spectrophotometry has been optimized and validated for the simultaneous extraction and determination of organic acids present in Iraqi calyces of Hibiscus Sabdraffia Linn. The method is based on using ultrasonic bath for extracting organic acids. Limit of detection in µg/ml of Formic acid, Acetic acid, Oxalic acid, Citric acid, Succinic acid, Tartaric acid, and Malic acid 126.8498×10^{-6}, 113.6005×10^{-6}, 97.0513×10^{-6}, 49.7925×10^{-6}, 84.0753×10^{-6}, 92.6551×10^{-6}, and 106.1633×10^{-6} ,respectively. The concentration of organic acids found in dry spacemen of calyces of Iraqi Hibiscus Sabdraffia Linn. under study: Formic acid, Acetic acid, Oxalic acid, Citric acid, Succinic acid, Tartaric acid, and Malic acid are 114.896 µg/g, 64.722 µg/g, 342.508 µg/g, 126.902 µg/g, 449.91 µg/g, 268.52 µg/g, and 254.07 µg/g respectively.

Key words: Organic acids, Hibiscus Sabdraffia Linn., RPHPLC- Uv/Vis.

Introduction

Hibiscus Sabdraffia Linn. is a tropical plant and belongs to malvaceae family[1]. The calyces are important part of Hibiscus Sabdraffia Linn. which dried under sun light and air, contain organic acids (tartaric, citric, malic, and hibiscic) showing high stability under the mentioned drying process[2]. Many phytochemicals have been found to be protective preventive against and many degenerative diseases and pathological processes such as in ageing, coronary heart disease, Alzheimers disease, neurodegenerative disorders, atherosclerosis cataracts, and inflammation [3]. Medical uses of Calyces are a wide, for example, infusion of calyces are regarded as diuretic, choleretic, febrifugal, hypotensive, decreasing the viscosity of blood and stimulating intestinal peristalsis [4], reduce hypertenstion [5]. Infusions of calyces are used as antiseptic, aphrodisiac, astringent, cholagogue, demulcent, diuretic, emollient, purgative, refreshment, sedative, stomachic, and tonic, traditionally [6]. The red calyces contain oxalic acid, tartaric acid, citric acid, malic acid [7].

Organic acids are typical products of microbial metabolism. All organic acids occur naturally in a variety of vegetable and animal substrates and can, therefore, be either naturally present as constituents of foods as a result of normal biochemical metabolic processes[8]. Among phytochemicals, organic acids may contribute to the protection against various diseases, due to the antioxidant potential [9]. Organic acids are such effective food preservatives because, apart from their antimicrobial inhibitory activities, they also act as acidulants and in so doing reduce bacterial growth by lowering the pH of

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food products to levels that inhibit bacterial growth [10-11].

The literature reviews, show that several methods have been developed for the determination of organic acids, spectrophotometric[12-13], enzymatic method[14], gas chromatography[15-17], capillary zone electrophoresis[18-22], and HPLC[23-27]. All the above method publications concentrated on the determination of organic acids in fruits and different beverages. There is no any publication about the simultaneous separation, qualitative, and quantitative determination of carboxylic acids in calyces by HPLC technique; so, we believe that the proposed method is the first procedure for the determination of the organic acids showed in Fig.1

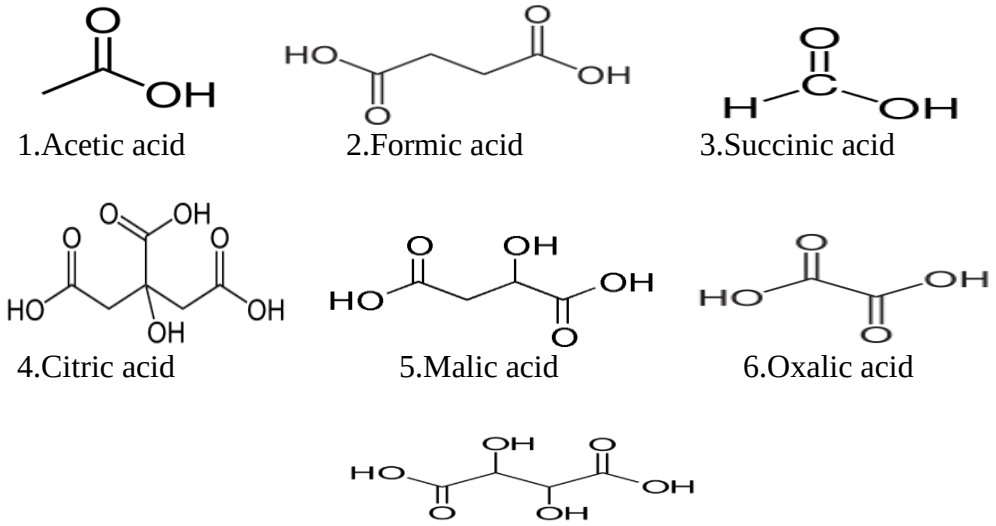

Fig.1: Structure of organic acids under study.

Materials and Methods:

1. HPLC Analysis.

The quantitative and qualitative analysis of organic acids were performed using Shimadzu HPLC system model LC-6A equipped with a binary pumps as solvent delivery model LC-10A from Shimadzu Corporation. The system is equipped with a Shimdzu SPD-6A ultraviolet – visible variable wave length (190-800 nm) detector. 20 µl samples was injected, and the chromatographic separation was performed on a RP-C_{18} Shimpack IC-A1 (3 µm) column, 50mm × 4.6 mm. After optimizing the instrumental and sample parameter, the HPLC analysis condition for the sample were 1.2 mM Potassium hydrogen phosphate at pH 4.5 as mobile phase, 1.0 ml/min as flow rate, and using 210 nm as maximum wavelength.

2. Chemicals.

All chemical reagents used for analysis organic acids were analytical Grade (99.99%) of BDH Company. The reagents include Potassium hydrogen phosphate, Deionized water, and Methanol. Standards of (1) acetic acid (99%), (2) formic acid (98%), (3) succinic acid (98%), (4) citric acid (99.8%), (5) malic acid (99.8%), (6) oxalic acid (99.9%), and (7) tartaric acid (99.9%) were purchased from BDH Company.

3. Hibiscus Sabdariffa Linn. sample.

Fresh Hibiscus Sabdariffa Linn. fruits were harvested from Baghdad, Iraq. After harvesting the fruits were washed with deionized water three times to clean them from dust, then seeds were

removed to obtain fresh Hibiscus Sabdariffa Linn. calyces. The calyces were dried at a room temperature for 7 days and then they Dried were immediately packed in polyethylene jars. Thereafter, the calyces also dried again in oven at 35 C° for three hours until constant weight. The dried calyces were grounded for 10 min using Agate mortar. The sample powder were immediately packed in polyethylene jars and kept in refrigerator until used.

4. Preparation of Standard Solution.

A standard stock solution of 1000 ppm of each organic acid was prepared by dissolving (1 g) in 1000 ml deionized water. A working standard solution of 25 ppm was prepared. For studying the optimization parameters, a standard working solution containing all organic acids with stable concentration was prepared.

5. Extraction Procedure.

Calyces liquid extracts were carried out by using deionized water: methanol solutions in different ratio, Table (1), were used for the extraction. Calyces powder (1 g) was placed in glass beakers, and 10 ml of extracting agent was added. The beakers were placed in the Ultra sonic bath (supplied from Karl Kolb, Germany) for 15 min, and then the mixtures were allowed to stand for 5 min. The supernatant was filtered through Whatman paper No.2 followed through micro filter (0.45 μm). The filtrate was kept in the glass tubes and preserved in refrigerator at 8 C°.

Table (1) Deionized water- methanol ratio used in extraction.

Samples	Deionized water- methanol ratio	
	Deionized water%	Methanol%
Sample 1	100	0
Sample 2	80	20
Sample 3	60	40
Sample 4	50	50
Sample 5	40	60
Sample 6	20	80
Sample 7	0	100

Results and Discussion:

1. Optimization Conditios

1.1. The Effect of pH.

The effect of pH of mobile phase in RP-HPLC for the separation a mixture of seven carboxylic acids were intensively studied. To five volumetric flasks (25 mL), containing 25 ppm of each carboxylic acid understudy, 1.2 mM potassium hydrogen phosphate (pH 2.5) was added, 0.5 M potassium hydroxide (1, 2, 3 and 4 mL) added to four volumetric flasks and added to all volumetric flasks deionized water to complete the volume and obtain PH (2.5, 3.5, 4.5, 5.5 and 6.5) respectively. The results obtained are shown in Table 2.

Table 2: Controlling of pH of mobile phase.

Organic acid	*PH*	*Retention time (min.)*	*Peak area (μv)*
Acetic acid	2.5	4.512	26291
	3.5	3.493	22678
	4.5	2.98	32062
	5.5	2.007	43261
	6.5	0.987	44451
Formic acid	2.5	5.975	28454
	3.5	4.39	22160
	4.5	3.823	30850
	5.5	3.018	47749
	6.5	2.152	45240
Succinic acid	2.5	7.147	28050
	3.5	5.573	21055
	4.5	5.155	24380
	5.5	3.5	52595
	6.5	2.82	46331
citric acid	2.5	9.95	32802
	3.5	7.823	15265
	4.5	6.075	23182
	5.5	4.163	47939
	6.5	3.882	51452
Malic acid	2.5	10.97	36143
	3.5	9.805	16817
	4.5	7.333	28191
	5.5	5.845	54743
	6.5	4.97	65344
Oxalic acid	2.5	12.962	24384
	3.5	11.068	19045
	4.5	8.415	28783
	5.5	6.84	49044
	6.5	5.95	58692
Tartaric acid	2.5	13.98	31473
	3.5	11.87	20450
	4.5	8.982	25635
	5.5	7.232	56334
	6.5	6.312	75565

Table 2 and Figure 2 show obviously that reversed phase separation can be

performed successfully at low pH value, because the low pH results is good solubility of the sample components and formation anions of organic acid molecules ($RCOO^-$) and form ion suppression of residual silanol groups on the silica matrix. The pH 4.5 was selected as the best one because in this medium of mobile phase with PH 4.5, it typically ensures the proper interaction of analytes with the non-polar, hydrophobic particle surface of C_{18} bonded silica (ODS) column.

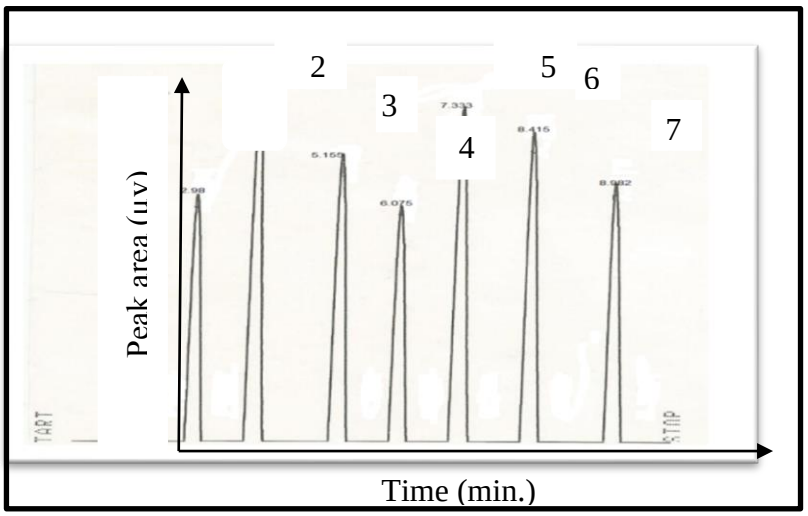

Fig. 2: Typical chromatogram of separating of organic acids in pH 4.5, the sequence of the peaks is as follow: 1. acetic acid, 2. formic acid, 3. succinic acid, 4. citric acid, 5. malic acid, 6. oxalic acid, and 7. tartaric acid.

The retention time data at different pH values between (2.5-6.5) show that, the organic acids have high retention time at low pH (2.5) due to the presence of undissociated COOH, So that the retention decreases with increasing the pH values, and when the pH and PI (Isolectric point) become coincident a local minimum occurs.

Fig. 3 shows that the relationship between the peak area and pH values which explain the general approach to the separation of the mixture containing an ionizable carboxylic acids is to suppress their ionization. Suppression of the ionization decreases a power of the molecular salvation and exposes the hydrophobic (organic) part of the molecule to the surface interaction. Ionization suppression is usually made by the adding of a buffer into the solvent which shift a PH to the certain value.

In the absence of the buffer, easy ionizable compounds are eluted from the column as very broad peaks, so, it was found that high pH values cause broad and tailed peaks, so, the peak area increased, and for these reason, pH 4.5 provides a good separation which can be noticed from the symmetrical shape of the peaks as well as good resolution.

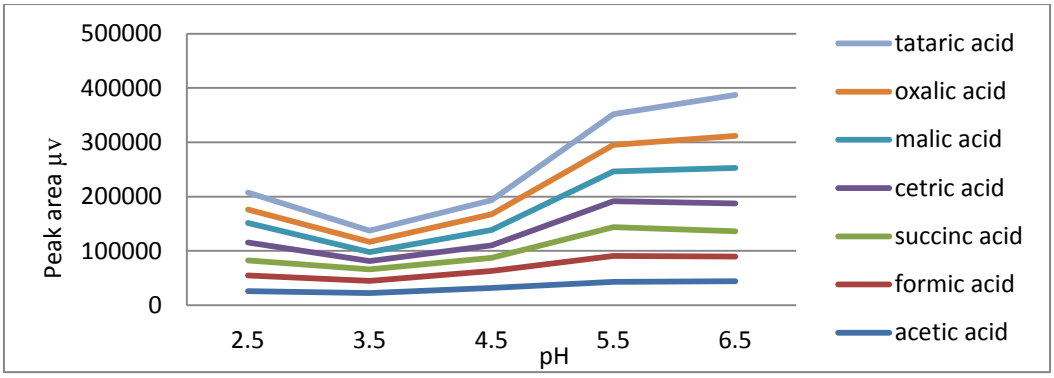

Fig. 4: The Effect of pH on peak area of organic acids separation using phosphate buffer solution.

1.2.Optimization of Flow Rate

Flow rate impacts HPLC system pressure chromatographic quality and analysis time. So, one must choose a flow rate that is appropriate for HPLC system and column. A higher than usual flow rate may adversely affects the quality of the chromatography not giving the analyte sufficient time to interact with the stationary phase. Faster is not always better. A lower than usual flow rate may leave the analyte waiting for the peak to appear at the detector. Therefore, the effects of flow rate were intensively studied as shown in Table 3.

Fig. 4 shows retention time and peak area as a function of flow rate of 1 ml.min^{-1} which demonstrates quite obviously a typical separation and resolution of carboxylic acids was achieved and leads to decrease solvent consumption and decrease in run time.

Table 3: The Effect of flow rate on retention time and peak area.

Organic acids	*Flow rate (ml.min^{-1})*	*Retention time (min.)*	*Peak area (µv)*
Acetic acid	0.6	1.733	92691
	0.8	1.59	66931
	1.0	1.398	59279
	1.2	1.237	33145
	1.4	1.133	47675
Formic acid	0.6	2.568	94464
	0.8	2.427	56625
	1.0	2.22	41765
	1.2	2.073	27486
	1.4	1.985	35545
Succinic acid	0.6	3.895	129953
	0.8	3.742	60811
	1.0	3.557	31193
	1.2	3.405	29324
	1.4	3.297	31941
Citric acid	0.6	4.825	113099
	0.8	4.667	46255
	1.0	4.475	39149
	1.2	4.322	41719
	1.4	4.223	23420
Malic acid	0.6	6.072	107084
	0.8	5.98	40154
	1.0	5.73	32164
	1.2	5.587	35882
	1.4	5.48	43224
Oxalic acid	0.6	7.145	102385
	0.8	6.978	35002
	1.0	6.815	31433
	1.2	6.662	25571
	1.4	6.555	29586
Tartaric acid	0.6	7.717	98943
	0.8	7.583	38271
	1.0	7.387	33574
	1.2	7.235	29792
	1.4	7.148	29968

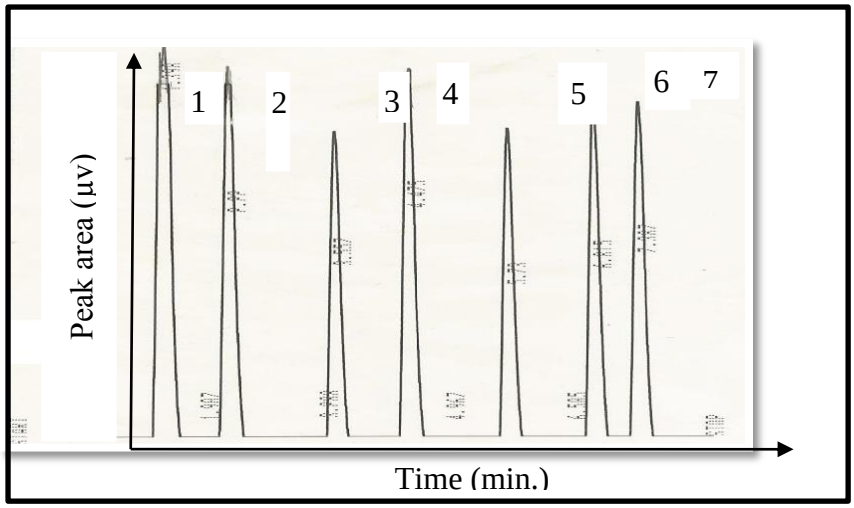

Fig. 4: Typical chromatogram of separation organic acids using a flow rate of 1 ml.min^{-1}, the sequence of the peaks is as follow: 1. acetic acid, 2. formic acid, 3. succinic acid, 4. citric acid, 5. malic acid, 6. oxalic acid, and 7. tartaric acid.

Fig. 5 shows the relationship between the peak area as a function of flow rate which confirms once again what has been achieved in Figure 6 that the flow rate of 1 ml.min^{-1} is the most appropriate flow rate because at lower flow rate lower than 1 ml.min^{-1} the area of each peak is very high and seem to be out of scale while at flow rate higher than 1 ml.min^{-1}, the area of each peak decreased dramatically, so the selection a flow rate of 1 ml.min^{-1} ensures stable base line , good resolution and retention time.

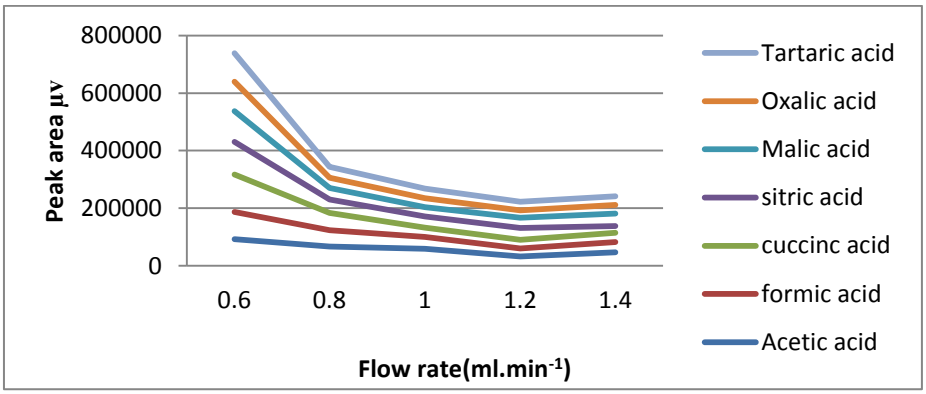

Fig. 7: The Effect of flow rate on separation organic acids as a function of peak area.

1.3. The Effect of Extraction Mixture on Extraction of Organic acids.

Seven kinds of solvent extracts from calyces of H. Sabdraffia Linn. were used to examine the effects of extraction solvent mixtures on organic acid concentration. All calyx extracts are rich source in organic acids but the best extraction occurs in 60% deionized water: 40% methanol as extraction agent. Figure 8 shows the best separation.

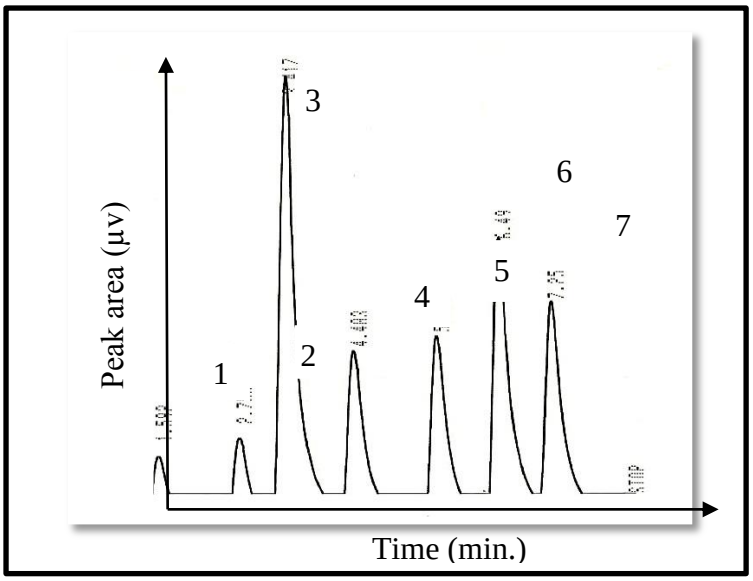

Fig. 8: Best separation of organic acids in calyces sample.

According to the above figure, the retention times, and the sequence of the peaks is as follow: 1. Acetic acid, 2. Formic acid, 3. Succinc acid, 4. Citric acid, 5. Malic acid, 6. Oxalic acid, and 7. Tartaric acid

Validation Method:

The validation study for acetic acid, formic acid, succinic acid, citric acid, malic acid, oxalic acid, and tartaric acid using RP-HPLC-UV/Vis was performed under the optimized conditions at 210 nm as maximum wavelength, 1.0 ml/min as flow rate of mobile phase, and 1.2 mM Potassium hydrogen phosphate at pH 4.5 as a mobile phase during analysis time (8 minutes).

1. Linearity and Limit of Detection (LOD).

Six standards solutions of organic acids were prepared in the following concentration: 0.78, 1.62, 3.25, 6.25, 12.5, and 25in µg/mL. The calibration curve obtained by plotting the peak area of chromatograms for 0rganic acids against the concentration, with three replicates (n = 3). Table 4 shows the validation of analytical method obtained from the calibration curves of organic acids analysed on RP-HPLC-UV/Vis with the linearity range (0.78-25 µg/mL)

Table 4: Validation of analytical method for organic acids by RP-HPLC-UV/Vis.

Organic acids	***Linear equation***	***R^2***	***LOD in ppm***	***RSD% n=3***	***Sensitivity in µv/ppm***
Formic acid	Y=2270.4x+4535.8	0.9953	126.8498×10^{-6}	0.009965	2270.4
Acetic acid	Y=2535.2x+5534.7	0.9955	113.6005×10^{-6}	0.010458	2535.2
Oxalic acid	Y=2967.5x+644.19	0.9994	97.0513×10^{-6}	0.010604	2967.5
Citric acid	Y=5784x-108.36	0.9981	49.7925×10^{-6}	0.005536	5784
Succinc acid	Y=3425.5x+5218.6	0.9973	84.0753×10^{-6}	0.008316	3425.5
Tartaric acid	Y=3108.3x+3187.7	0.9932	92.6551×10^{-6}	0.007801	3108.3
Malic acid	Y=2712.8x+2088.9	0.993	106.1633×10^{-6}	0.011569	2712.8

2. Mean concentration of organic acids in Calyces of Iraqi Hibiscus Sabdraffia Linn.

Table 4 shows the mean concentration of organic acids for the best separation from the calyces extract in ratio (60% deionized water: 40% methanol). Triplicate measurement was done on the best extract sample.

Table 4: Mean concentration of organic acids.

Organic Acid	*Mean concentration ±SD (ppm)*
Formic acid	11.4896± 2.0
Acetic acid	6.4722± 1.9
Oxalic acid	34.2508± 2.1
Citric acid	12.6902± 2.2
Succinc acid	44.991± 1.8
Tartaric acid	26.852± 1.7
Malic acid	25.7035± 2.0

3. ***Concentration of Organic acids in Dry Spacemen of Calyces of Hibiscus Sabdraffia Linn.***

Table 5 shows the concentrations of the substances which was studies by this research in the dry spacemen of calyces.

Table 5: Concentration of organic acids.

Substance	*Concentration (µg/g)*
Formic acid	114.896
Acetic acid	64.722
Oxalic acid	342.508
Citric acid	126.902
Succinc acid	449.91
Tartaric acid	268.52
Malic acid	254.07

4. Interference study

To investigate effect of other organic acids on measurements, standard addition method was carried out. It was observed that lactic acid (2 mg/ mL), propanoic acid (1 mg/ mL), valeric acid (2 mg/ mL), fumaric acid(2mg/ mL), and benzoic acid(2 mg/ mL) did not affect the measurement of the acids under study and showed a different retention time.

Conclusions

The proposed analytical method by RP- HPLC- Uv/ Vis for simultaneous separation and determination of seven organic acids naturally present in Hibiscus sabdraffia calyces was highly convenient for evaluation the level concentration of the compounds under study. The obtained results show also, that the Iraqi Hibiscus Sabdraffia calyces are rich in these compounds.

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فصل وتقدير بعض الحوامض العضويه في زهره نبات الكجرات العراقي الجافه

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الخلاصه:
طريقة كرموتوغرافيا السائل عالية الأداء- طور معاكس الجديدة (RP-HPLC) مع مطيافية الأشعة فوق البنفسجية- المرئية (Ultraviolet- Visible spectrophotometry) تم أمثلتها وأثبتت لأستخلاص والتعيين في وقت واحد الحوامض العضوية الموجودة في زهرة نبات الكجرات العراقية.
الطريقة أسست على أستخدام حمام مائي فوق صوتي لأستخلاص الحوامض العضوية. عوامل النموذج والجهاز تم أمثلتها بطريقة الأمثلة التقليدية (عامل واحد كل مرة). حدود التحسس لمضادات الأكسدة وهي بوحدة µg/ml:
Formic acid, Acetic acid, Oxalic acid, Citric acid, Succinic acid, Tartaric acid, and Malic acid 126.8498×10^{-6}, 113.6005×10^{-6}, 97.0513×10^{-6}, 49.7925×10^{-6}, 84.0753×10^{-6}, 92.6551×10^{-6}, and 106.1633×10^{-6} respectively
تركيز الحوامض العضوية التي تم دراستها لنموذج زهرة نبات الكجرات الجافة هي كالاتي:
Formic acid, Acetic acid, Oxalic acid, Citric acid, Succinic acid, Tartaric acid, and Malic acid are 114.896 µg/g, 64.722 µg/g, 342.508 µg/g, 126.902 µg/g, 449.91 µg/g, 268.52 µg/g, and 254.07 respectively.

الكلمات المفتاحية: حوامض عضوية، نبات الكجرات.