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Simultaneous Determination of the Main Organic Acids in Anatolian Black Pine by HPLC with DAD Detector

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The versatile characterization of organic materials in Anatolian black pine (*Pinus nigra*) especially the significant organic acids is great importance. A rapid, simple, and sensitive HPLC–DAD method was developed and presented for a simultaneous detection of six organic acids. This method was thoroughly validated to determine the most abundant organic acids present in Anatolian black pine and their distribution in branches, leaves and cones. Separation of six organic acids were developed and achieved on ODS C_{18} column (250mm×4.6mm, 5µm particle size), using a mixture of (A) water (pH: 2.0, adjusted by H_2SO_4) and (B) acetonitrile in the ratio of (95:5, v/v) at wavelength of 210 nm with a flow rate of 0.8 mL min^{-1} within 14 minutes. The dynamic range was between 5 to 1000 mg L^{-1} with relative standard deviation less than 0.73%, (n=4). Limits of detection and the recoveries for oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid were ranged 1.85 mg L^{-1} (87.75-99.36%), 3.61 mg L^{-1} (100.89-119.55%), 4.01 mg L-1 (83.57-119.64%), 1.16 mg L-1 (82.29-112.19%), 1.19 mg L-1 (80.86-110.42%) and 2.87 mg L-1 (96.96-117.74%), respectively.

Keywords: Anatolian black pin, organic acids, RP-HPLC-DAD, optimization and validation method, simultaneous detection

INTRODUCTION

Black pine (*Pinus nigra*) is belonging to Pinaceae family which grown in centre, west and south regions of Anatolia, is one of the most widely grown tree in Turkey [1,2,3,4]. Black pine is growing naturally in 20% of all forested areas in Turkey and one of the most common medicinal plants in Turkey which its turpentine has been used for several years in Turkish folk medicine due to antiseptic effects on respiratuar system and urinary diseases [2, 3, 4, 5]. Additionally, it is used for back pain as resin plaster and as stomachical, dermatological, and analgesic drugs [2]. In fact, black pine has an economic importance in pharmaceutical and cosmetic sectors [3]. Recently studies have found that the pine species extract contains flavonoids, condensed polymers, phenolic acids and organic acids (including: oxalic acid, lactic

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acid, acetic acid, tartaric acid, formic acid, citric acid, malonic acid, succinic acid, fumaric acid, glutaric acid and malic acid) [5, 6, 7, 8, 9, 10].

Organic acids are important constituent for many plants playing a role in many metabolic reactions to contribute on plant growth and can accumulate in large amounts as dissolved free anions [7, 10, 11, 12]. Organic acids can bind elements such as metals, and their role in whether & soil processes as detoxification agents [7]. Higher concentrations of organic acids have been found in heavy metal tolerant plants than in sensitive plants [7, 10, 13]. Aluminium has been found to increase production of organic acids by roots, especially oxalic acid, malic acid and citric acid [7,10]. Citric acid has been shown to be an effective Al chelator and has been proposed to decrease Al toxicity [7]. Aguiar et al. [14] had provided that oxalic acid & tartaric acid inhibit Fe^{+3} reduction and avoiding $OH^{\bullet}$ radical formation. Ahoen-Jonnarth et al. [7], Hsieh et al. [8] and Kuo et al. [9] had reported that oxalic acid was a dominant most abundant of organic acids in different pine species extract. The success in clarification of these functions depends on the identification of organic acids in different parts of plants [15]. The concentration levels of organic acids in pine extracts were variable [7]. The studies and data on the size distributions of organic acids in the pine spesies are very limited and how they change during ripening [7,9,16].

Analysis of organic acids by HPLC has become increasingly important due to their role in the physiological activity of plants such as citric acid and oxalic acid are parts of Krebs cyclic pathway (carbohydrates, lipids and proteins) [11]. A numerous publications and research papers focused on separation methods to detect the most of these organic acids for simultaneous determination using RP-HPLC in fruits, vegetables, plants, soil, sediment, food and wine [15,16,17,18,19,20,21,22]. On other hand, a seldom publications focused on detect these organic acids in pine species using RP-HPLC or GC [7,9,10,12]. The present study was the first academic report interesting to determine the size distributions of six organic acids including (oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid) (see Fig. 1) in main three parts of Anatolian black pine (branches, leaves and cones) which were collected from Bozok University forest (Yozgat, Turkey).

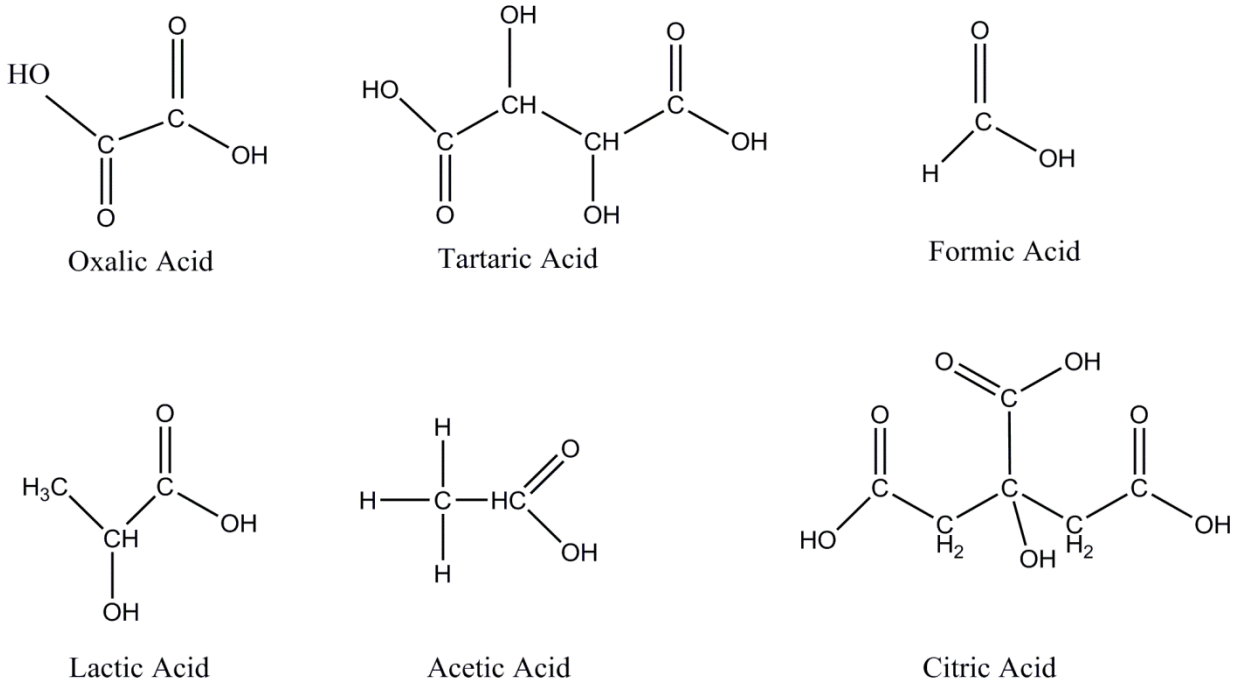


Figure 1. Strcutures of main organic acids in Anatolian black pine.

As well as, the analytical method was validated for the simultaneous detection of six organic acids in Anatolian black pine using HPLC with DAD detector.

MATERIALS AND METHODS

Reagent and materials

Working standard of six organic acids were laboratory grad and obtained from Merck (Darmstadt, Germany) with purity ≥ 98%. Standard working solutions was prepared by diluting appropriate amounts of the standard samples in water and keep it in freezer away from light (stock solutions). HPLC grade of acetonitrile (99.99%) was purchased from BDH Prolabo (Australia). While Sulphuric acid and phosphoric acid were purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, sodium chloride and sodium hydroxide were analytical grad obtained from Carlo Erba (Milano, Italy). Reverse-osmosis ultrapure type quality water used was obtained from Millipore water purification system (DIRECT-Q 8UV, USA).

Collection of Anatolian black pine samples

Three main parts of Anatolian black pine (branches, leaves and cones) were collected between 03rd to 04th of April, 2015 from Bozok University forest (Yozgat, Turkey). From each tree, 3 branches of 5 cm diameter were cut. 1.5-2 kg of black pine samples (branches, leaves and cones) were taken from one tree.

Extraction of organic acids from Anatolian black pines samples

Branches and cones of Anatolian black pine were dried in sunlight and ground to a fine powder. Extraction of organic acids from Anatolian black pine parts (branches, leaves and cones) was performed according to method described by Gode et al. [13] and Hees et al. [23] with slight modification. 50 g of leaves were cleaned with deionized water then dried and ground in an iron mortar. Subsequently, 400 mL of NaOH (0.1 mM) firstly was added and the mixture was placed in refrigerator at 4 ^{o}C for 24 hours. Secondly, sample was placed in ultrasonic bath for 30 minutes then filtered and washed with 50 mL of NaCl (5%, w/v) and shaken for 3 minutes then filtered. Finally, sample was rinsed twice in 25 mL Millipore water. This cleaning procedure was very important to ensure that all organic acids were removed from Anatolian black pine sample to extracted solution. A blank was run and no interfering peaks were found. The extracted solution (500 mL) was acidified by means of H_3PO_4 to pH 5.0 then filtered through a 0.48 μm syringe filter.

Standard preparation

The stock solutions of oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid were freshly prepared and dissolved in 25 mL water in a brown volumetric flask. Then gradually diluted to obtain seven different concentrations (5, 10, 100, 250, 500, 750 and 1000 mg L^{-1}) and all standards were stored at 4 °C until use.

Chromatographic analysis

The quantitative analysis of six organic acids were performed on Shimadzu (Kyoto, Japan) HPLC autosampler system model LC-20AT equipped with four pumps and a SPD-M20A diode array detector (DAD). 20 μL samples was injected and the

chromatographic separation was performed on a Inertsil C_{18} ODS-3 column (5µm particle size, 4.6 mm×250 mm, Japan).

Analysis was isocratic at λ_{max}:210 nm, 60 ˚C (column temperature) with flow rate of 0.8 mL min^{-1} using a mixture of (A) water (adjusted to pH 2.0 with H_2SO_4) and (B) acetonitrile as mobile phase at (95:5, v/v) level (total retention time was 14 minutes). The mobile phase (A) was prepared freshly everyday and filtered through a 0.45 µm membrane filter to remove any particulate matter then degassed by sonication before use. The sensitivity of the detector was set at 0.01 AUFS. Prior to injecting samples, the column was equilibrated at least for 30 min with the mobile phase flowing through the system. Each sample was injected in triplicate, with the relative standard deviation (R.S.D) below 0.73% and 9.82% for standard samples and real samples, respectively.

Optimization of chromatographic conditions

In order to achieve the optimum separation for simultaneous determination of six organic acids, following conditions were studied: (I) Mobile phase composition using two different binary mixtures (water, pH 2.0:ACN), (KH_2PO_4 ,4 mM:ACN) varied from 100:0 to 0:100 with column temperature and flow rate kept constant at 40˚C and 0.8 mL min^{-1}, respectively. (II) Mobile phase (A) water pH varied at 1.0, 2.0, 2.5, 3.0, 4.0, 5.0 and 7.0 (adjusted by H_2SO_4), with column temperature and flow rate kept constant at 40°C and 0.8 mL min^{-1}, respectively. (III) Flow rate was varied from 0.2 to 1.8 mL min^{-1} with mobile phase (A) water, pH and column temperature maintained at 2.0 and 40˚C, respectively. (V) Column temperature was varied from 10 to 75 ˚C with mobile phase (A) water, pH and flow rate fixed at 2.0 and 0.8 mL min^{-1}, respectively. Moreover, the effects of different level of all these factors were systematically addressed on system suitability parameters such as resolution factor, theoretical plates, retention time and asymmetry factor.

Validation of the developed method

The proposed method was validated in the light of (International Conference on Harmonization) ICH Guidelines [24,25] for linearity, accuracy, sensitivity, specificity and robustness. Consequently, the following were performed.

Linearity

Linearity of an analytical method was established by automatic injections of the standard mixture solutions in the investigated ranges from low to high concentrations, each concentration was repeated four times. Seven different concentrations of six organic acid as described previously and calibration curve was constructed in the specified concentration range. The calibration plot (peak area ratio of each organic acid versus its concentration) was generated by replicate analysis (n = 4) at all concentration levels and the linear relationship was evaluated using the least square method within Microsoft Excel program.

Accuracy

The accuracy of the method (recovery) was assessed by adding two know amount of the six organic acids to a sample solution of know concentration and was expressed in terms of the percent recovery. The recovery rate of oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid at two different fortification levels (50 and 500 mg L^{-1}) was evaluated in order to assess the extraction efficiency of the proposed method. For this, 50 g of branches or leaves or cones were spiked

with 50 and 500 mg L^{-1} of mix stock solution of six organic acids were then mixed with 400 mL of NaOH (0.1 mM). Resulting samples were mixed and allowed to stand for 24 hours in refrigerator at 4 °C before extractions. Four replicates at each fortification level were prepared.

Sensitivity

The instrumental response sensitivity is the slope of the calibration line because a method with a large slope is better able to discriminate between small differences in analyte content. Limit of detection (LOD) and limit of quantitation (LOQ) were determined according to following equation [24]:

$$LOD \text{ or } LOQ = k (B/S) \qquad \text{Equation 1}$$

where k is a constant (3 for LOD and 10 for LOQ), B is the standard deviation of the analytical signal, and S is the slope.

Specificity

Specificity of the proposed method was evaluated by peak purity curves through resolution factors (R_s), peak asymmetry factor (A_s) and number of theoretical plates (N). The resolution factor R_s was calculated based on equation 2 [11]:

$$R_s = (t_2 - t_1) / (W_2/2 + W_1/2) \qquad \text{Equation 2}$$

Where t_1 and t_2 are the retention times of the two components, W_1 and W_2 are the corresponding widths at the bases of the peaks obtained by extrapolating the relatively straight sides of the peaks to the baseline. The asymmetry factor is a measure of peak tailing and was calculated based on equation 3 [26]:

$$A_s = b/a \qquad \text{Equation 3}$$

Where A_s is peak asymmetry factor, b is the distance from the point at peak midpoint to the trailing edge and a is the distance from the leading edge of peak to the midpoint (a and b were measured at 10% of peak height). The number of theoretical plates (N) were calculated using equation 4 [26]:

$$N = 16 \times (t_R/W_1)^2 \qquad \text{Equation 4}$$

Where, N is the number of theoretical plates, t_R is retention time and W_1 is width at the bases of the peak.

Robustness

The robustness of the method was determined by making slight changes in the chromatographic conditions, such as flow rate, column temperature, and buffer pH.

RESULTS AND DISCUSSION

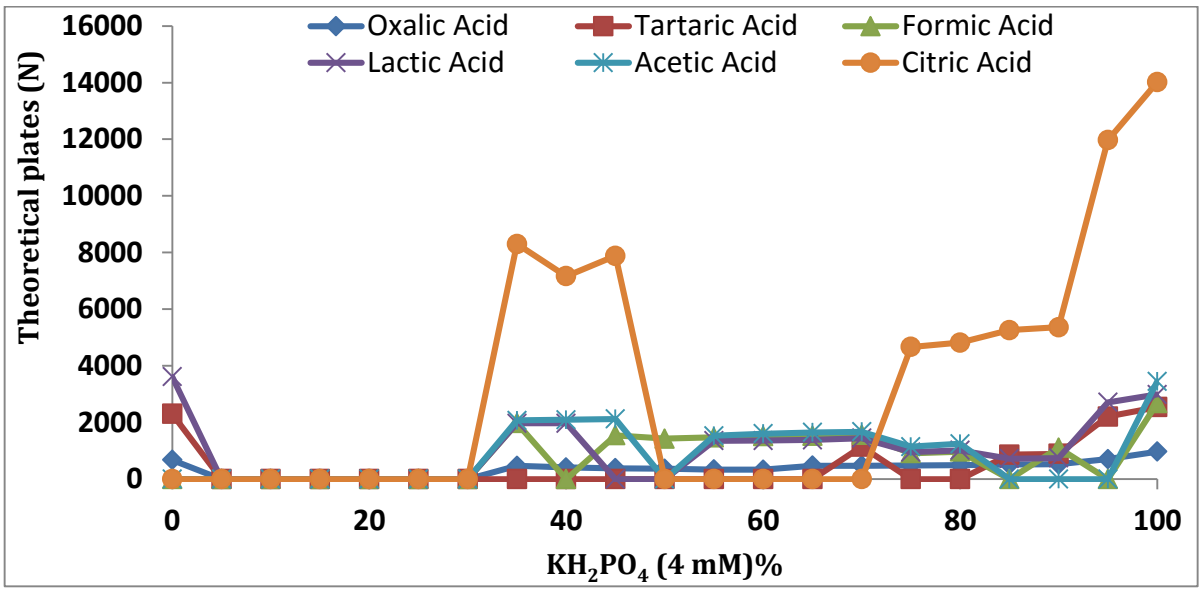
Optimization of chromatographic conditions

The effects of different chromatographic conditions on the instrumental responses create a situation where one has to compromise between different experimental variables in order to achieve the best chromatographic separation.

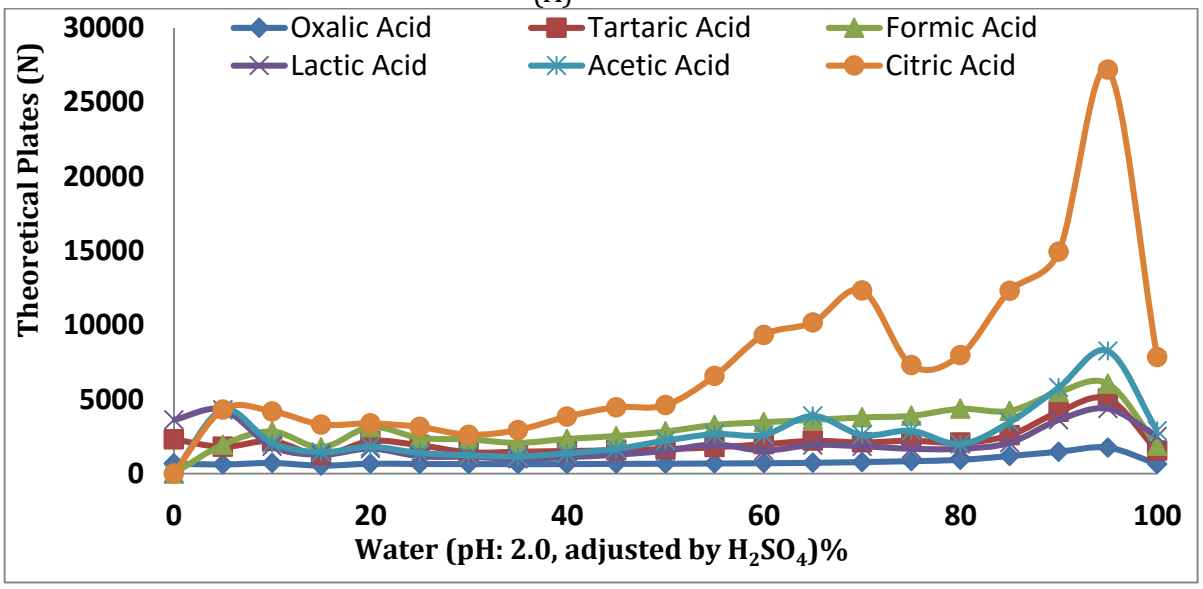
Effect of mobile phase composition

The optimum mobile phase composition was based on comparison between the most common binary mixture which were performed to analysis organic acids and used on this proposed method to find the most suitable parameters for simultaneous detection of oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid in Anatolian black pine at λ_{max}:210 nm. The first binary mixture

was (A) KH_2PO_4 (4 mM) with (B) acetonitrile which reported by Shi et al. [10] in pinus radiata, Zhang et al. [15] in wine, mulberry vinegar, apple & orange and Scherer et al. [27] in fruits & juices. The second binary mixture was (A) water (pH: 2.0, adjusted by H_2SO_4) with (B) acetonitrile which had been reported by Kelebek et al. [28] in orange juice and orange wine and Castellari et al. [29] in grape musts and wines. The ratio of both binary mixtures were started from (A:B, 100:0, v/v) to (A:B, 0:100, v/v) levels at the flow rate of 0.8 mL min^{-1} and column temperature 40 °C is shown in Fig. 2.



(A)



(B)

Figure 2. Effect of mobile phase composition (A) KH_2PO_4 with acetonitrile and (B) water (pH: 2.0, adjusted by H_2SO_4) with acetonitrile on the number of theoretical plates of oxalic acid, taratric acid, formic acid, lactic acid, acetic acid and citric acid using RP-HPLC-DAD at λ_{max}= 210 nm.

Addition of appropriate amount of acetonitrile to the mobile phase as phase B can reduce the retention time of six organic acids and improve the peak shapes to a certain extent. Minimum retention times of six organic acids were obtained using (A) water (pH: 2.0, adjusted by H_2SO_4), with (B) acetonitrile as mobile phase at

(95:5, v/v) level, which makes the method rapid and a one of the most desirable criteria. Though retention time was shorter, satisfactory resolution ($R_s \geq 1.5$) and asymmetry values were achieved ($A_s \leq 1.15$). An adequate theoretical plates (~ 27000) is indicative of a good column performance. Fig. 2 (A) (KH_2PO_4 (4mM): ACN) as mobile phase showed that the asymmetry was ($A_s > 1.9$) at (90:10, v/v) and still higher at (0:100, v/v) which indicates wide tailing of the peaks, but was ($A_s < 1.8$) at (100:0, v/v) to (95:5, v/v). On other hand, as can be seen from Fig. 2 (B) water (pH: 2.0, adjusted by H_2SO_4): ACN) as mobile phase, the asymmetry was ($A_s > 1.5$) at (85:15, v/v) and still higher at (0:100, v/v) which indicates tailing of the peaks, but was (As < 1.4) at (90:10, v/v).

Effect of mobile phase pH

The optimization of mobile phase pH was achieved on water using H_2SO_4 at varied pH values (1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0 and 7.0), the remaining three factors were kept constant, i.e. mobile phase composition (water: ACN; 95:5, v/v), flow rate of 0.8 mL min^{-1} and column temperature of 40 °C. The changes in retention times, number of theoretical plates and asymmetry factors for six organic acids were enumerated in Table 1.

As shown in the Table 1 (except at pH 7), retention times and asymmetry factors decrease with the increase in pH while the number of theoretical plates increases. The number of theoretical plates was maximum at pH 2.0. The dissociation constant (pK_a) of oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid are 1.25, 2.89, 3.77, 3.86, 4.76 and 3.13 at (25 °C), respectively. According to this values six organic acids will be unionized at pH 2.0, 2.5, 3.0 and 3.5 with using

Table 1: Effect of mobile phase pH values on retention time (t_R), number of theoretical plates (N) and asymmetry factor (A_s) of six organic acids

pH		Oxalic acid	Tartaric acid	Formic acid	Lactic acid	Acetic acid	Citric acid
1.0	t_R	3.37	3.61	0	0	0	9.91
	N	2019.0	5505.6	0	0	0	24165.0
	A_s	1.10	0.95	0	0	0	1.15
2.0	t_R	3.51	3.84	4.05	6.32	6.71	10.39
	N	2063.6	5727.5	6256.8	6875.7	7772.2	26363.1
	A_s	1.05	1.10	1.15	1.10	1.10	1.10
2.5	t_R	3.53	3.88	4.29	6.34	6.72	10.68
	N	1842.5	5416.9	6055.9	6496.4	7318.2	22647.9
	A_s	1.25	1.30	1.35	1.40	1.40	1.35
3.0	t_R	3.61	3.89	4.18	6.38	6.75	10.86
	N	565.8	1196.8	1667.0	1620.9	1831.8	6093.9
	A_s	1.40	1.65	1.50	1.45	1.45	1.50
3.5	t_R	3.67	4.34	4.85	6.43	6.82	10.99
	N	440.7	713.1	1045.9	1186.5	1344.2	4260.6
	A_s	1.50	1.75	1.75	1.60	1.60	1.65
4.0	t_R	3.98	0	0	6.48	6.84	11.13
	N	313.1	0	0	923.2	1050.7	2366.8
	A_s	1.70	0	0	1.78	1.80	1.80
5.0	t_R	4.65	0	0	6.53	6.88	11.46
	N	286.0	0	0	601.2	672.3	2239.7
	A_s	1.90	0	0	1.95	1.95	2.00
7.0	t_R	1.78	0	0	0	4.94	8.10
	N	34.9	0	0	0	389.9	867.1
	A_s	1.50	0	0	0	1.65	1.75

octadecylsilane (ODS, C_{18}) column because of the less interaction between organic acids and C_{18} under ionized condition. However, when pH values were at 4.0, 5.0 and 7.0 for tartaric acid and formic acid, at 1.0 and 7.0 for lactic acid and at 1.0 for formic acid and acetic acid could not reach baseline separation which does not have much affinity for the ionized hydrophilic for these organic acids. At lower pH value, the ionized hydrophilic species are not much portioned with stationary phase and hence gives symmetric peak. The resolution was poor at pH 2.5, 3.0 and 3.5 ($R_s \leq 1.5$) for all organic acids but was highest at pH 2.0 ($R_s \geq 1.5$). Looking at the importance of the different chromatographic parameters, pH 2.0 was found to be optimum.

Effect of mobile phase flow rate

Mobile phase flow rate was studied at varied values (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6 and 1.8 mL min^{-1}) and was enumerated in Table 2.

From Table 2, it can be observed that theoretical plates were highest at flow rate of 0.8 mL min^{-1} with asymmetry factors less than 1.15. The change in flow rate had slight significant effect on resolution factor while retention time decreased as the flow rate increased (untill flow rate of 1.2 mL min^{-1}). The flow rate exceeded 1.0 mL min^{-1}, peaks from several organic acids started to overlap (tartaric acid, formic acid, lactic acid and acetic acid) with R_s less than (1.5).

Table 2: Effect of mobile phase flow rate on retention time (t_R), number of theoretical plates (N) and asymmetry factor (A_s) of six organic acids

mL min^{-1}		Oxalic acid	Tartaric acid	Formic acid	Lactic acid	Acetic acid	Citric acid
0.2	t_R	17.06	18.01	18.77	28.07	29.77	31.81
	N	1163.6	2306.5	2505.3	3890.7	4367.5	11243.1
	A_s	1.85	1.95	1.95	1.90	1.90	1.95
0.4	t_R	8.58	8.97	9.59	14.65	15.56	18.25
	N	1455.5	3576.8	4088.3	4241.8	4781.9	14806.0
	A_s	1.65	1.70	1.75	1.70	1.70	1.70
0.6	t_R	5.75	6.12	6.49	10.08	10.72	13.68
	N	1468.4	3742.9	4213.3	4515.8	5110.3	18711.5
	A_s	1.35	1.40	1.45	1.40	1.40	1.40
0.8	t_R	3.52	3.79	4.01	6.29	6.76	10.39
	N	2202.7	5745.6	6432.1	6944.4	7885.4	27635.7
	A_s	1.05	1.10	1.15	1.10	1.10	1.10
1.0	t_R	3.17	3.46	3.65	5.83	6.23	9.82
	N	1787.6	4780.3	5340.7	6048.7	6904.5	24706.8
	A_s	1.25	1.35	1.40	1.35	1.35	1.30
1.2	t_R	2.92	3.22	3.35	5.34	5.69	8.98
	N	1626.6	4152.5	4483.6	5421.0	6157.4	23368.6
	A_s	1.45	1.60	1.60	1.55	1.55	1.55
1.4	t_R	2.51	2.75	2.86	4.57	4.87	8.21
	N	1379.4	3346.9	3632.9	5341.9	6064.0	20371.9
	A_s	1.60	1.75	1.75	1.70	1.75	1.70
1.6	t_R	2.20	2.41	2.51	4.0	4.26	7.24
	N	1145.6	2715.2	2942.9	4489.0	5041.0	16575.7
	A_s	1.70	1.80	1.85	1.80	1.75	1.75
1.8	t_R	1.95	2.14	2.22	3.56	3.79	6.47
	N	974.4	2263.6	2444.7	3516.49	3981.6	13851.1
	A_s	1.75	1.80	1.85	1.80	1.80	1.80

Effect of column temperature

Seven different column temperatures (10, 20, 30, 40, 50, 60 and 75 ˚C) were tested to study the effect of temperature on separation of six organic acids as shown in Table 3.

The shortened retention time with higher theoretical plates for all six organic acids when column temperature was increased. The results in table 3 showed that tartaric acid and formic acid at (75˚C) could not reach baseline separation. For fast and accurate detection, the optimum column temperature of 60˚C was chosen for this study with acceptable resolution factor (more than 1.5) and highest number of theoretical plates.

Validation of the the proposed method

After the chromatographic method had been developed and optimized, it must be validated. The validation of an analytical method verifies that the characteristics of the method satisfy the requirements of the application domain.

Linearity (calibration curve)

The linearity of analytical procedure of six organic acids was constructed by spiking seven different concentrations for each organic acid (5, 10, 100, 250, 500, 750, 1000 mg L^{-1}) with four replicates and evaluated by plotting detector response (peak area) versus organic acid concentration (mg L^{-1}) to obtain the calibration curve and correlation coefficient (R^2). The chromatographic responses were found to be linear over an analytical range of 5–1000 mg L^{-1} and found to be quite satisfactory and reproducible with time. The linear regression equation was calculated by the least squares method using Microsoft Excel program and summarized in Table 4.

Table 3: Effect of column temperature on retention time (t_R), number of theoretical plates (N) and asymmetry (A_s) of six organic acids

˚C		Oxalic acid	Tartaric acid	Formic acid	Lactic acid	Acetic acid	Citric acid
10	t_R	3.92	4.24	4.52	6.64	7.17	11.11
	N	985.4	1796.9	2045.7	2826.8	3292.0	16147.9
	A_s	1.75	1.85	1.85	1.75	1.75	1.85
20	t_R	3.81	4.07	4.38	6.50	7.02	10.92
	N	1094.7	1832.7	2130.5	2817.2	3286.8	17529.8
	A_s	1.60	1.75	1.75	1.65	1.65	1.70
30	t_R	3.65	3.97	4.13	6.35	6.86	10.63
	N	1335.2	2058.6	2224.6	3183.9	3404.6	20088.3
	A_s	1.25	1.45	1.45	1.30	1.35	1.35
40	t_R	3.53	3.84	4.08	6.29	6.69	10.37
	N	2074.6	5614.0	6039.5	6587.1	7451.6	26460.4
	A_s	1.05	1.10	1.15	1.10	1.10	1.10
50	t_R	3.50	3.81	3.99	6.27	6.66	10.36
	N	2325.2	5806.4	6364.8	6988.9	7885.4	27476.4
	A_s	1.05	1.05	1.5	1.05	1.05	1.10
60	t_R	3.47	3.76	3.95	6.23	6.63	10.32
	N	2461.6	6279.3	6908.2	7388.9	8365.3	28394.3
	A_s	1.00	1.05	1.10	1.05	1.05	1.05
75	t_R	3.42	0	0	5.72	6.16	9.07
	N	2223.9	0	0	5818.6	6739.3	21073.7
	A_s	0.90	0	0	1.05	1.05	1.00

Table 4: Regression analysis of calibration curves for six organic acids by proposed method.

Organic Acid	Liner range (mg L^{-1})	t_R (minutes)	Calibration Equation	R^2	RSD %	LOD (mg L^{-1})	LOQ (mg L^{-1})
Oxalic acid	5 – 1000	3.47	y = 10134x - 15589	0.9997	0.59	1.85	5.61
Tartaric acid	5 – 1000	3.74	y = 1613.6x + 2896.4	0.9998	0.51	3.61	10.94
Formic acid	5 – 1000	3.96	y = 884.87x – 3342.2	0.9996	0.53	4.01	12.16
Lactic acid	5 – 1000	6.23	y = 423.18x –456.33	0.9997	0.65	1.16	3.51
Acetic acid	5 – 1000	6.63	y = 627.14x –510.89	0.9997	0.69	1.19	3.61
Citric acid	5 – 1000	10.32	y = 1119.2x –28339	0.9997	0.73	2.87	8.69

t_R: retention time, **R^2**: coefficient of determination, **RSD**: relative standard deviation, **LOD**: limit of detection, **LOQ**: limit of quantitation.

Table 4 showed that the correlation coefficient were found to be ≥ 0.9996 with R.S.D ≤ 0.73 and R_s ≥ 1.5, indicating a strong linear relationship between the variables and suggesting that the developed HPLC-DAD method had an excellent linearity (see Fig. 3).

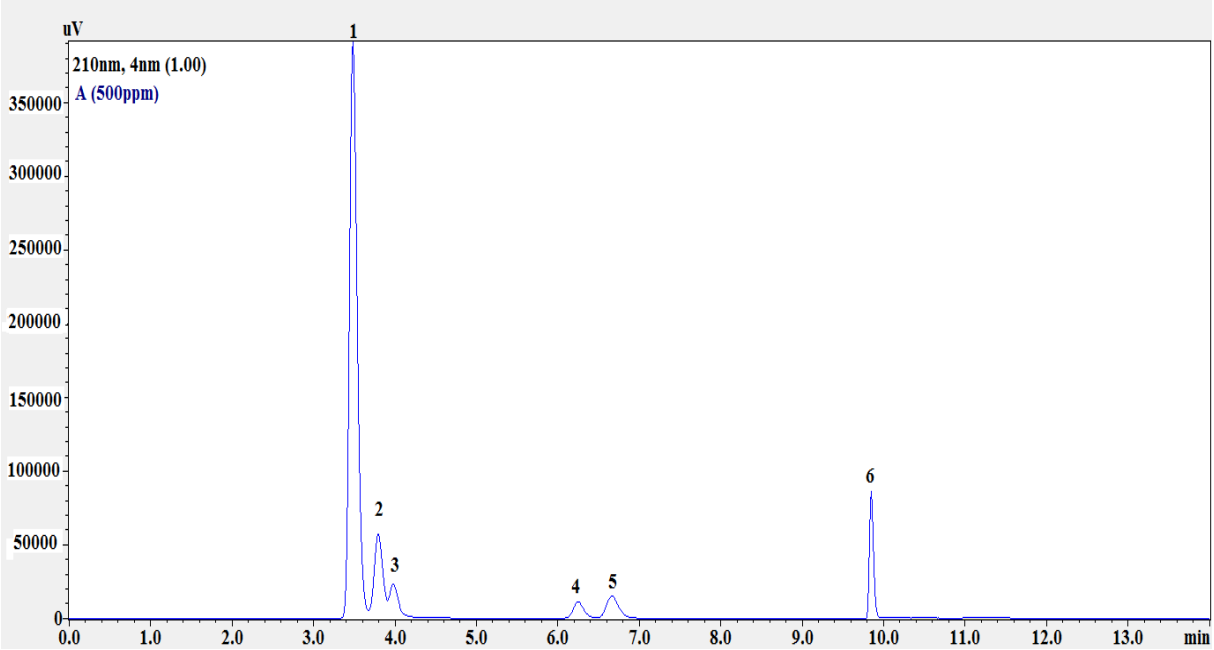
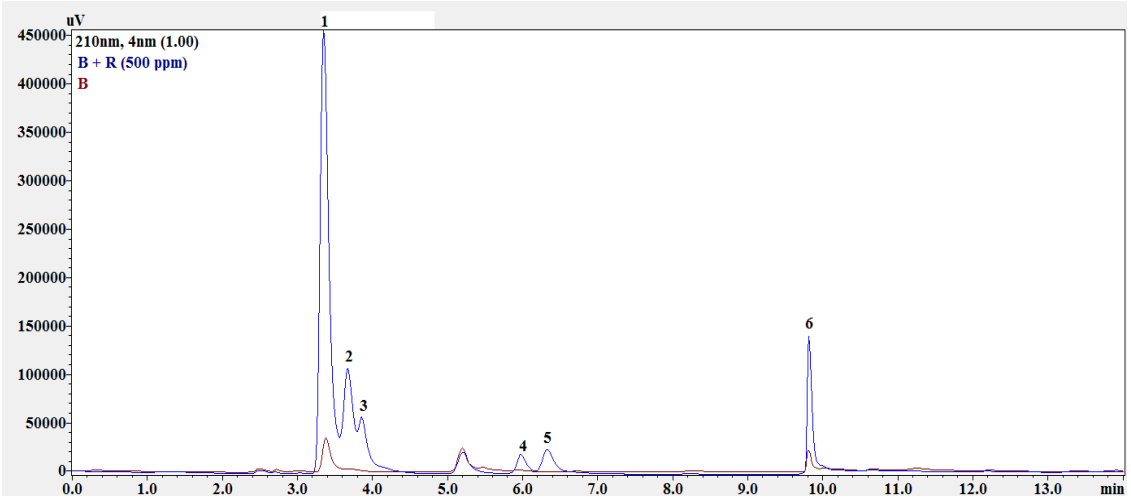


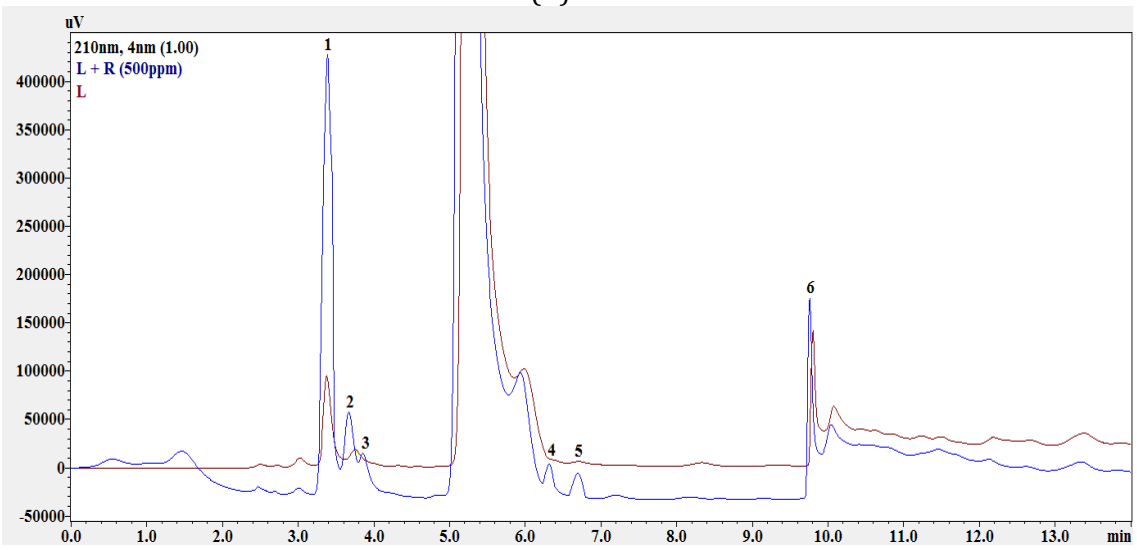
Figure 3. Typical chromatogram of oxalic acid (1), tartaric acid (2), formic acid (3), lactic acid (4), acetic acid (5) and citric acid (6), (500 mg L^{-1}) using H_2SO_4 (pH:2.0) with acetonitrile as mobile phase and flow rate 0.8 mL min^{-1} at (λ_{max}: 210 nm) with column oven temperature 60°C by RP-HPLC-DAD.

Accuracy (Recovery)

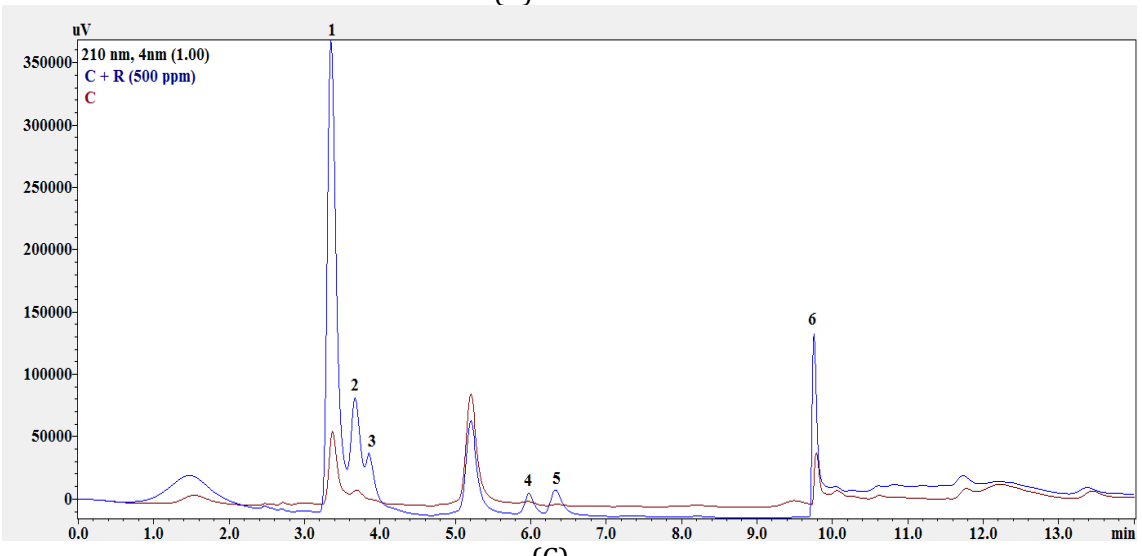
The accuracy of the proposed method was performed to verify the effectiveness of the extraction step. The accuracy study on this developed method was achieved using the technique of standard addition at two spiked levels 50 and 500 mg L^{-1}, 12 samples from the same Anatolian black pine tree for each part (branches, leaves and cones) 4 samples were collected. Two of four samples for each part were saved as control, while the other two samples were added standard mixture of six organic acids at two different amounts (50 and 500 mg L^{-1}) as seen in Fig. 4.



(A)



(B)



(C)

Figure 4. Chromatogram of recovery efficiency and organic acids distrubtion (oxalic acid **1**, tartaric acid **2**, formic acid **3**, lactic acid **4**, acetic acid **5** and citric acid **6**) in extracts of Anadolu *pinus nigra* branches (A), leaves (B) & cones (C) using H_2SO_4 (pH:2.0) with acetonitrile as mobile phase and flow rate 0.8 mL/min at (λ_{max}: 210 nm) with column oven temperature 60°C by RP-HPLC-DAD.

The recovery ranges in this study for formic acid, acetic acid and citric acid (83.57-119.64, 80.86-110.42 and 96.96-117.74%, respectively) are better than previous paper reported by Rodrigues et al. [30] which were (76, 112 and 122 respectively) using the external standard addition methodology. While, the recovery ranges of oxalic acid, tartaric acid and lactic acid in this study (87.75-99.36, 100.89-119.55 and 82.29-112.19%, respectively) were similar with earlier study reported by Cunha et al. [18] (99.6-112.9%) for tartaric acid using the external standard addition methodology and by Kakola et al. [21] (96.2-111.4 and 94.5-103.5%) for oxalic acid and lactic acid. Thus, good recoveries with R.S.D below than 10 were obtained for each organic acid, confirming that the developed method was accurate.

Sensitivity

LOD_s for (oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid) by RP-HPLC-DAD in this study (1.85, 3.61, 4.01, 1.16, 1.19 and 2.87 mg L^{-1}, respectively) were lower compared with previous publications. Cunha et al. [18] reported that LOD_s of tartaric acid, lactic acid, acetic acid and citric acid in fruit juices & nectars were (50.00, 25.00, 17.00 and 60.00 mg L^{-1}, respectively), Shui and Leong [19] reported that LOD_s of lactic acid, acetic acid and citric acid in fruit juices were (9.64, 10.91 and 5.93 mg L^{-1}, respectively), Rodrigues et al. [30] reported that LOD_s of formic acid, acetic acid and citric acid in green coffee were (7, 34 and 20.00 mg L^{-1}, respectively), Chinnici et al. [31] reported that LOD_s of oxalic acid and citric acid in fruit juices were (2.00 and 3.30 mg L^{-1}, respectively), Zhang et al. [15] reported that LOD_s of lactic acid, acetic acid and citric acid in several complex liquid biological systems were (2.45, 2.69 and 3.54 mg L^{-1}, respectively), Eyeghe-Bickong et al. [32] reported that LOD_s of tartaric acid and citric acid in grapevine berries were (20.00 and 30.00 mg L^{-1}, respectively) and. Thus, the LOD_s for (oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid) using RP-HPLC-DAD in this study were better compared to previous studies.

Extraction efficiency of real samples of Anatolian black pine

The developed method was applied to detected the distribution of six organic acids in main three parts (branches, leaves and cones) of Anatolian black pine and summarized in Table 5.

It was found that the concentration level of oxalic acid, formic acid, acetic acid and citric acid in leaves extract of Anatolian black pine in this study (667.04, not detected, not detected and 223.63 mg kg^{-1}, respectively) were lower compared with the concentration level of oxalic acid, formic acid, acetic acid and citric acid (2024.00, 239.50, 1.46 and 485.00 mg kg^{-1}, respectively) in leaves extract of pine morrisonicola Hayata as reported by Kuo et al. [9] While the concentration level of tartaric acid and lactic acid (75.11 and 54.82 mg kg^{-1}, respectively) were higher copmared with the same study (26.30 and not detected mg kg^{-1}, respectively). The most abundant organic acid in main three parts of Anatolian black pine was oxalic acid which was similar with pine sylvestris as reported by Ahoen-Jonnarth et al. [7] and pine morrisonicola Hayata as reported by Kuo et al. [9] Thus, the heavy metal levels in Anatolian soil (Yozgat, Turkey) lower than Fushui village (Yuchih, Taiwan).

Table 5: Results of recovery efficiency and organic acids distribution in extracts of Anatolian *Pinus nigra* branches, leaves and cones.

Organic Acid		Control±SD (mg/kg)	RSD %	Added (mg/L)	Found±SD (mg/L)	RSD %	Recovery %
Pinus nigra branches	Oxalic acid	289.26±4.02	1.45	50	49.68±1.73	0.22	99.36
				500	404.27±1.96	0.86	90.79
	Tartaric acid	N.D	-	50	58.68±0.13	0.10	117.36
				500	597.76±10.59	1.63	119.55
	Formic acid	N.D	-	50	43.36±3.78	0.98	86.72
				500	598.22±14.06	1.23	119.64
	Lactic acid	40.10±1.27	2.60	50	42.81±0.82	0. 87	85.62
				500	503.63±7.90	1.42	100.73
	Acetic acid	N.D	-	50	46.78±0.21	0.45	93.57
				500	552.13±17.05	3.08	110.42
	Citric acid	162.14±1.05	0.64	50	56.18±0.76	0.68	112.36
				500	484.81±26.17	4.46	96.96
Pinus nigra leaves	Oxalic acid	667.04±5.16	0.99	50	43.88±4.13	2.37	87.75
				500	457.78±15.08	2.66	91.55
	Tartaric acid	75.11±2.72	3.65	50	55.75±2.29	3.64	111.49
				500	514.84±27.10	5.26	102.97
	Formic acid	N.D	-	50	41.79±3.73	9.81	83.57
				500	437.32±35.69	8.23	87.46
	Lactic acid	54.82±0.98	1.86	50	49.87±4.32	7. 03	99.75
				500	560.96±28.73	5.07	112.19
	Acetic acid	N.D	-	50	40.43±1.62	3.93	80.86
				500	451.43±10.80	2.33	90.29
	Citric acid	223.63±3.97	2.06	50	55.97±6.27	6.50	111.94
				500	538.46±30.67	4.99	107.69
Pinus nigra cones	Oxalic acid	568.18±21.04	4.76	50	46.00±2.34	2.31	92.00
				500	480.46±17.10	3.19	96.09
	Tartaric acid	60.13±5.73	9.18	50	50.57±1.88	3.22	101.14
				500	504.44±4.36	0.85	100.89
	Formic acid	N.D	-	50	54.56±4.57	9.01	109.12
				500	535.48±2.91	0.55	107.09
	Lactic acid	90.14±1.26	1.56	50	41.15±2.75	5.38	82.29
				500	490.11±24.59	4.92	98.02
	Acetic acid	68.43±2.37	3.87	50	42.23±1.14	2.28	84.45
				500	450.40±17.39	3. 80	90.08
	Citric acid	117.29±3.31	8.92	50	52.02±6.43	7.22	104.04
				500	588.72±9.51	1.52	117.74

***N.D**: not detected (below than LOD).*
***SD**: standard deviation (n =4).*

CONCLUSION

In the present work, a rapid, simple, convenient and sensitive reversed phase HPLC method has been developed, optimized and validated for simultaneous monitoring of (oxalic acid, tartaric acid, formic acid, lactic acid, acetic acid and citric acid) in Anatolian black pine (branches, leaves and cones) using DAD detector with isocratic elution. Optimization showed that the mobile phase composition and pH are more crucial parameters to be controlled than flow rate and column temperature for reproducible and quantitative estimation of these organic acids. The short overall time of sample analysis of 14 minutes cuts down and thereby makes the method more cost effective. The present method was selective enough to analyze six organic acids simultaneously in Anatolian black pine by the absence of any interfering peaks from other coexisting endogenous substances at the retention time of each organic acids and without any tedious sample clean-up procedure. In summary, the optimized chromatographic estimation of six organic acids with good resolution in a short time can be used for applied to routine therapeutic monitoring of these organic acids.

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