

Development And Validation Of The Conductometric Titration Method Of Quantitative Determination Of Free Organic Acids In The Anise Fruits

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ABSTRACT: *A conductometric titration method has been developed for determination of free organic acids in anise fruits. The direct acid base titration of free organic acids was carried as a titrant was used the standardized sodium hydroxide aqueous solution. The end-point of titration was fixed by a conductometric electrode. The calibration curve was found to be linear in the concentration range of 0.0112 - 0.0332 mg/cm³ ($r^2 = 0.9992$). The percentage recovery was found to be in the range of 98.61 - 101.25%. The intra-day and inter-day precision were 1.32% and 1.62%, respectively. The method was found to be accurate and precise, having relative standard deviation of less than 2%. The developed method was validated statistically by compliance with SPhU guidelines. The proposed method is simple, convenient, rapid and sufficiently precise and can be successfully applied for the quantitative determination of total free organic acids in anise fruits.*

Keywords: *Anisum vulgare, organic acids, conductometric titration, validation*

1. INTRODUCTION

The use of herbal medicines in modern medicine and pharmacy not only remains stable, but tends to a certain increase, while drugs of natural and synthetic origin harmoniously complement each other in the fight against various human diseases. At the same time, many medicinal plants are used not only in medicine, but also in the perfumery-cosmetic, food industry.

Anise (*Anisum vulgare* Gaertn.) – an annual herbaceous plant belonging to the umbelliferous family (*Apiaceae*) is one of such plants with centuries of experience in medical use. It is currently widely cultivated in many countries of Southern Europe, North Africa, Asia Minor and Mexico. It blooms in June-July, the fruits ripen in August. The fruits of this plant are well studied and are a source of essential oil. Its main component is anethole (80-90%); a significant proportion is methylchavicol (10%). In addition, the oil contains anisic aldehyde, anisketone and anisic acid. The seed kernel contains a lot of fatty oil (10-25%).

It has been established that anise essential oil has bronchodilating [1], anticonvulsant [2], antifungal [3] and insecticidal [4] properties.

In turn, the fruits of this plant exhibit an antioxidant and anti-inflammatory [5] effect, the seeds have hepatoprotective [6], antimicrobial and antioxidant effects [7].

The plant organism is very complex both in its chemical composition and in the functions it performs. It should be noted that in nature, plants are the only creators of inorganic organic substances, without which life would be impossible for humans and animals. Plants have an amazing variety of metabolism and are able to synthesize a huge amount of various compounds from carbon dioxide, water and inorganic compounds. The fixation of atmospheric carbon dioxide in the absence of light is widespread in plants, during which the formation of biologically and pharmacologically active substances, namely organic acids.

Organic acids are compounds of an aliphatic or aromatic series, which are characterized by the presence in the molecule of one or more carboxyl groups. This class of compounds is widespread in the plant world. Organic acids are contained in plants mainly in the form of salts, esters, dimers, as well as in a free state, forming buffer systems in the plant cell sap.

Organic acids such as citrate, malate, and oxaloacetate play important roles in several major metabolic pathways in plants, including fatty acid and amino-acid biosynthesis, as well as in modulation adaptation to stressful conditions [8,9].

The composition of organic acids that accumulate varies depending on the species, cultivars, age of the plant and tissue type [10].

The organic acids that accumulate in high levels in plant tissue play potential roles as metabolically active solutes for osmotic adjustment and the balance of cations. Organic acids are also key components in the mechanisms that some plants use to cope with nutrient deficiencies [11].

Organic acids have a wide range of biological effects on the human body: in addition to vitamin properties, they have a choleric effect that normalizes the activity of the digestive system. Organic acids regulate the secretion of bile and pancreatic juice, improve appetite; have bactericidal properties and reduce the putrefactive processes in the body. Ascorbic acid, increasing the redox processes in tissues, plays an important role in a skin barrier function, promotes regeneration with various injuries by stimulating assimilation and dissimilation processes [12].

Nowadays analytical methods, such as titration, ion chromatography (IC) [13, 14], high-performance liquid chromatography-ultraviolet detection (HPLC-UV) [15], capillary electrophoresis (CE) [16], gas chromatography (GC) and thin-layer chromatography (TLC) [17] are commonly utilized to analyze organic acids.

Regulatory documentation of determining the content of free organic acids in terms of malic acid is offered a direct alkalimetry method with a mix of phenolphthalein and methylene blue until the moment of appearing a lilac color in the foam [18]. This determination is possible in presence of a weak extraction color and good foaming (rosehips, mountain ash, viburnum). In the case of intense coloration and/or if the foam is absent, the advantage belongs to potentiometry (fruits of black currant, peppermint, lemon balm, sage) [19]. As the extract of the fruits of anise has a brown color, and the foam is practically absent, potentiometric determination should be preferred. On the other hand, the potentiometric method of analysis

is more accurate and reliable, its main disadvantage is the duration of fixing the equivalence point due to the long-term establishment of a constant value of the potential after adding another portion of the titrant. Therefore, in the case of determining the equivalence point of titration of organic acids` solutions with low concentration, coulometric [20] or conductometric titration [21] are more economical in terms of time.

2. MATERIALS AND METHODS

2.1. Apparatus

A Hanna 2550 digital pH meter equipped with a conductometric electrode HI 76310 was used throughout the study. All titrations were carried out manually.

2.2. Reagents and materials

Malic acid, citric acid, ascorbic acid, oxalate acid, succinic acid, benzoic acid were purchased from Merck (Germany)

Sodium hydroxide (0.1 M): Accurately 2.0 g of the pure NaOH was dissolved in distilled water. The solution was made up to 500 mL with the same water and standardized.

n-butanol and *glacial acid* of analytical grade were purchased from Merck (Germany).

The TLC-plates silica gel 60 F254 were purchased from Sorbfil (Russia).

2.3. Procedures

2.3.1. Extraction of organic acids from anise fruits for TLC analysis

It was taken 5.0 g of a dry raw material, previously crushed to a particle size of 2-3 mm, than filled with 25 mL of water (1:5) and heated under reflux in a boiling water bath for 1 h. The resulting extract was filtered through a filter. The raw materials were extracted twice with new portions of the solvent, after that the extracts were combined.

2.3.2. Determining of organic acids in anise fruits using TLC

The chromatographic development was carried out in a chamber (19 × 19.5 × 6.5 cm). TLC-plates made of Sorbfil silica gel 60 F254 (15×15 cm) were used in experiments. The samples were spotted using a 10 µl micro-pipette with 5 µl of extract`s solution and standards solutions. Developing system was consisted of a mixture of n-butanol-glacial acid-water (4:1:2). A series of 10% (w/v) standard solutions containing equal concentration of citric acid, ascorbic acid, oxalate acid, succinic acid, benzoic acid, malic acid were prepared. The plates were sprayed by an indicator solution of 0,2 g of bromocresol green in 100 mL of 96% ethanol solution. The chamber was previously saturated by solvent system for 60 min. The development of the chromatogram was allowed to proceed until the solvent had traveled 11-12 cm beyond the starting line 20 min at room temperature. The TLC plates were then removed from the chamber and allowed to dry in air. The dried TLC plates were sprayed with the indicator solution, and the color was developed by brief heating 1–3 min in a hot dry oven (100° C).

2.3.3. Extraction of organic acids from anise fruits

It was taken 5.0 g (an exact amount sample) of the crushed raw material and placed in a 250 mL flask with ground glass joints, poured 200 mL of purified water and kept for 2 hours in a boiling water bath. After cooling, the solution was quantitatively transferred into a 250 mL volumetric flask and make up to the mark (solution A).

2.3.4. Titrimetric determination of amount of free organic acids in anise fruits

10 mL of solution A was placed in a titration cell and 250 mL of freshly boiled water was added. After that, the prepared solution was titrated with standardized sodium hydroxide aqueous solution using conductometric titration. After each addition of titrant, the solution was stirred magnetically for 30 s and the steady conductivity was noted. The addition of titrant was continued until no significant change in conductivity on further addition of titrant. In addition, a blank experiment was carried out, according to which the blank volume of 0.1 mole/L sodium hydroxide solution was 0.010 mL.

The equivalence point was determined by applying the graphical method. The graph was drawn by plotting volume of titrant on X axis and conductivity values on Y axis, which was expressed in μS . According to results, a conductometric titration curve was obtained due to direct proportion of conductivity to volume of titrant than the equivalence volume of titrant was determined by the tangent method (Figure 1).

The content of total amount of free organic acids in terms of malic acid in absolutely dry raw materials in percent was calculated according to following expression:

$$X = \frac{(V_{eq} - V_x) \cdot 0.00067 \cdot 250 \cdot 100 \cdot 100 \cdot K}{m \cdot 10 \cdot (100 - W)}$$

where, 0.00067 – the amount of malic acid, which is equivalent to 1 mL of sodium hydroxide solution (0.1 mole/L), in grams; V_{eq} is the volume of sodium hydroxide solution (0.1 mole/L), which was used for titration, in milliliters; V_x – volume of sodium hydroxide solution (0.1 mole/L), which was spent for titration in a blank experiment, in milliliters; m is the mass of raw materials in grams; K is correction coefficient for 0.1 mole/L sodium hydroxide solution; W is the loss in mass upon drying of the raw material as a percentage.

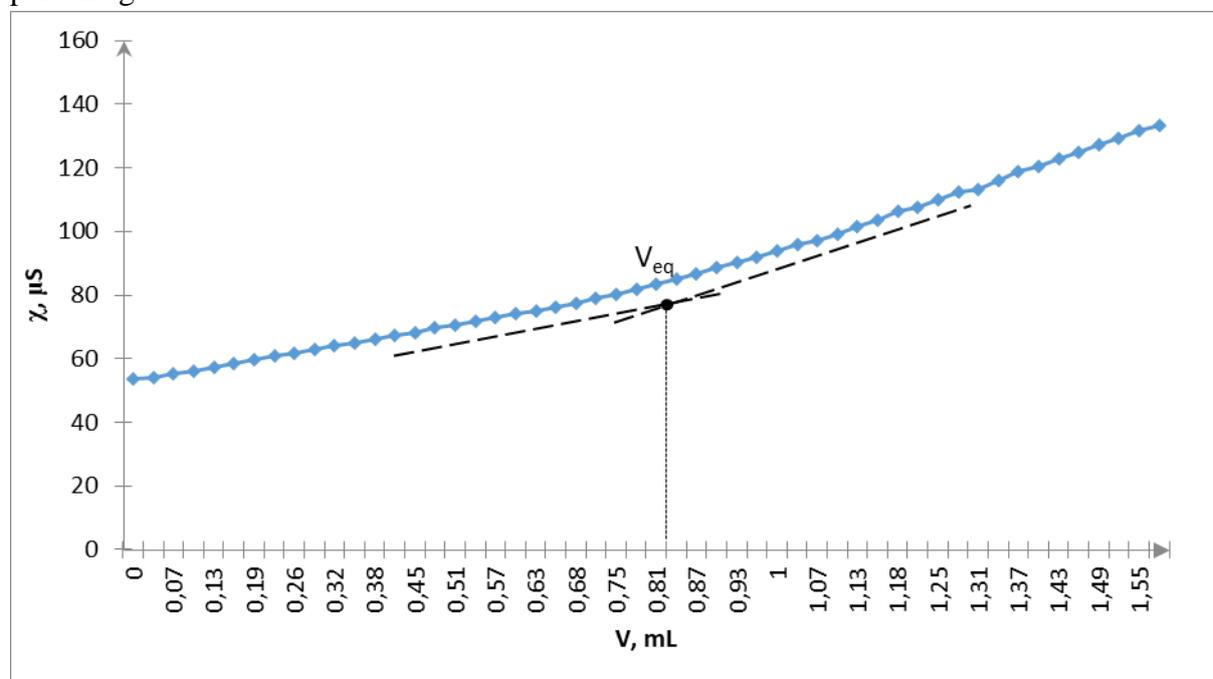


Figure 1. Conductometric titration curve of determination total free organic acids in anise fruits

2.4. Method validation

Conductometric method was validated in compliance with SPhU guidelines [22]. The following parameters were validated.

2.4.1. Precision

The precision of the conductometric methods was evaluated in terms of repeatability and intermediate precision (intra-day and inter-day, respectively). The repeatability was performed by the analysis of the raw material that was repeated for six times with the same concentration. The intermediate precision of the method was determined as described above under the conditions of the same laboratory, but in different days. The amount of free organic acids present in raw material was calculated and the % RSD was calculated.

2.4.2. Accuracy

In order to check the accuracy of the developed method, analytical recovery experiments were carried out by using standard addition method in three different concentrations 80%, 100% and 120%. From the total amount of free organic acids found, the percentage recovery was calculated. This procedure was repeated for three times for each concentration and the % RSD was calculated.

2.4.3. Robustness

Robustness of conductometric method was done at three different concentration levels 80%, 100% and 120% of free organic acids. Robustness was expressed as the RSD of the same procedure applied by two different analysts as well as using two different burettes.

2.4.4. Linearity

For the establishment of method linearity, eleven different weights of raw material corresponding to 50%, 60%, 70%, 80%, 90%, 100%, 120%, 130%, 140%, 150% of the about weight 5.0 g were taken and analyzed conductometrically. The values of correlation coefficient, slope and intercept were determined.

2.4.5. Specificity

The specificity of the method was studied by conductometric titration of a solvent and prepared solution of extract of anise fruits in an appropriate solvent: distilled water.

3. RESULTS AND DISCUSSIONS

3.1. Determination of organic acids in anise fruits using TLC

The spots of organic acids were colored in yellow color. The TLC separation of organic acids revealed 3 spots in anise fruits extracts, which identified by comparison of their R_f to those of reference organic acids as malic acid ($R_f = 0.58$), citric acid ($R_f = 0.51$), succinic acid ($R_f = 0.40$).

3.2. Validation

3.2.1. Accuracy studies

In order to check the accuracy of the developed method, known quantities of malic acid in three different concentrations were added to its pre analyzed sample and analyzed by the developed method. The percentage recovery was found to be in the range of 98.61 – 101.25%. The results of recovery studies are shown in Table 1. The % RSD value was found to be 1.04%. The low % RSD values is confirmed a high degree of correspondence between

the value of the calculated and obtained content of the total amount of free organic acids in the studied samples.

3.2.2. Precision

The precision of the method was confirmed by intra – day and inter – day analysis. The % RSD values for intra – day and inter – day analysis were found to be 1.32 and 1.62%, respectively. The % RSD values were less 2%, it is proved that the method is precision (Table 2,3).

3.2.3. Robustness

The robustness of the method was established by inter – analysts and inter – burettes. The RSD values of inter – analysts and inter – burettes analysis were found to be 1.48 and 1.29%, respectively. The RSD values were less than 2% for method, which proves a good robustness of the method (Table 4).

3.2.4. Linearity

The linearity is proved in the range of concentrations from 50 to 150%. Calibration curve was drawn by plotting concentration of free organic acids on X axis and volume of titrant values on Y axis (Figure 2). The regression equation of curve has the following form: $y = 35.714x + 0.0139$. The correlation coefficient value was equal 0.9992, which indicates the presence of direct proportion of volume of titrant to concentration of organic acids in the studied solutions within a given range of the method (Table 5).

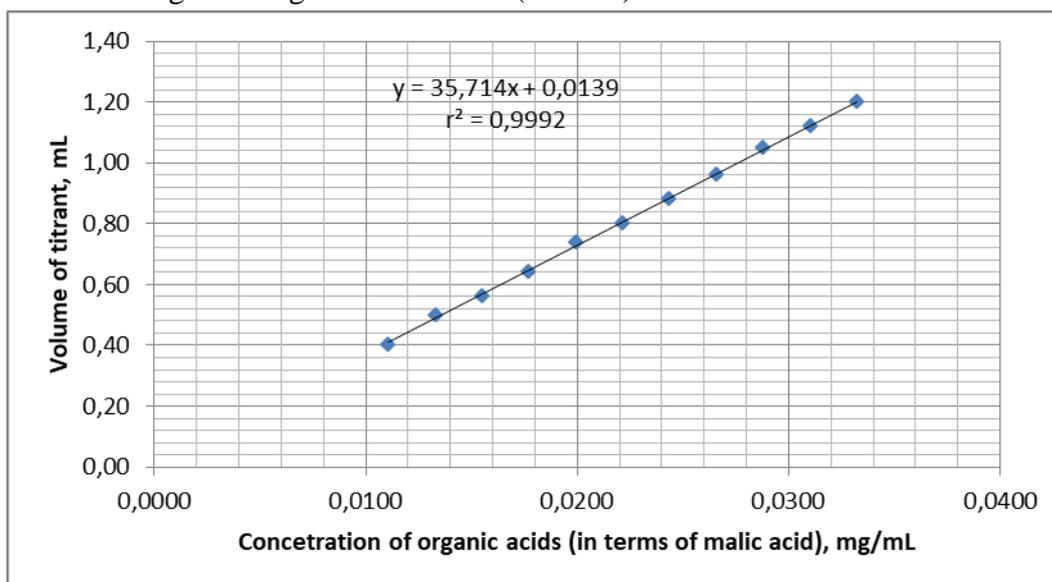


Figure 2. Calibration curve of volume of titrant in dependence to content of free organic acids (in terms of malic acid, mg/mL) in extracting of anise fruits

3.2.5. Specificity

The specificity of the method was established by analysis of the blank solution and prepared solution of extract of anise fruits. There was revealed neither interference of solvent nor probable impurities on result of quantitative determination of total free organic acids in anise fruits.

4. CONCLUSION

The thin-layer chromatography was allowed us to identify malic, citric, and succinic acids in anise fruits.

The developed conductometric titrimetric method is found to be simple, accurate, and precise as result, can be used for determination the total free organic acids in anise fruits. The developed method was validated as par SPhU guidelines. It is proved that the proposed method for the quantitative determination of organic acids is valid in respect of specificity, linearity, analytical area of the method, precision, accuracy and can be used for the quantitative determination of total free organic acids in anise fruits.

Table 1. Recovery studies by standard additions technique

Amount present (µg/mL)	Amount added (µg/mL)	Amount found (µg/mL)	Amount recovered (µg/mL)	Recovery %	SD %	RSD %
0.0222	0.0177	0.0398	0.0176	99.31	1.05	1.04
0.0222	0.0177	0.0399	0.0177	100.20		
0.0222	0.0177	0.0397	0.0175	98.61		
0.0222	0.0222	0.0446	0.0224	101.12		
0.0222	0.0222	0.0443	0.0221	99.38		
0.0222	0.0222	0.0447	0.0225	101.25		
0.0222	0.0266	0.0486	0.0264	99.43		
0.0222	0.0266	0.0485	0.0263	98.86		
0.0222	0.0266	0.0491	0.0269	101.22		

Table 2. Intra – day precision of organic acids in anise fruits by conductometry

Number of sample	Equivalent volume of titrant, mL
1	0.80
2	0.79
3	0.81
4	0.80
5	0.78
6	0.79
Mean, mL	0.80
SD, %	0.0104
Confidence interval, mL	0.0083
RSD, %	1.32

Table 3. Inter – day precision of organic acids in anise fruits by conductometry

Number of sample	Equivalent volume of titrant, mL	
	The first day	The second day
1	0.80	0.81
2	0.81	0.80
3	0,79	0.78
4	0.78	0.79

5	0.80	0.80
6	0.81	0.82
Mean, mL	0.80	0.80
SD, %	0.0117	0.0141
Confidence interval, mL	0.0094	0.0141
RSD, %	1.46	1.77
Mean RSD, %	1.62	

Table 4. Robustness of organic acids in anise fruits by conductometry

Concentration of organic acids, %	Equivalent volume of titrant, mL				RSD, %	
	The first analyst	The second analyst	The first burette	The second burette	Inter – analysts	Inter – burettes
80	0.64	0.64	0.65	0.63	1.48	1.29
80	0.66	0.65	0.64	0.62		
80	0.65	0.63	0.63	0.64		
100	0.82	0.80	0.80	0.81		
100	0.81	0.82	0.79	0.80		
100	0.79	0.81	0.81	0.79		
120	0.96	0.96	0.96	0.95		
120	0.98	0.95	0.97	0.94		
120	0.97	0.93	0.95	0.96		

Table 5. Results of linearity studies for conductometric method

№	Mass of raw material	Concentration		Equivalent volume of titrant
	g	mg/cm ³	%	cm ³
1	2.4998	0.0112	50	0.40
2	3.004	0.0134	60	0.50
3	3.5002	0.0156	70	0.56
4	4.0005	0.0177	80	0.64
5	4.5003	0.0200	90	0.74
6	5.0002	0.0222	100	0.80
7	5.5004	0.0244	110	0.88
8	6.0001	0.0266	120	0.96
9	6.5005	0.0288	130	1.05
10	7.0006	0.0310	140	1.12
11	7.4997	0.0332	150	1.20

Regression equation: $y = 35.714x + 0.0139$
Slope (b): 35.714, Intercept (a): 0.0139
Correlation coefficient, r^2 : 0.9992

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