Research Paper

Optimization of voltammetric technique for determination of fumaric and maleic acids in apple juice

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Abstract
The natural occurrence of butenedioic acid, fumaric and maleic acid isomers in apple juices at levels greater than 3 mg L^{-1} may be due to the metabolism of fungi or bacteria present in the apple itself. Fumaric acid and maleic acid are electrochemically reduced in acidic solutions (pH 1.20) at the same potential value, being determined in the form of butenedioic acid and in solutions with pH 8.20 it is possible to differentiate the two acids with the reduction taking place at different potentials. This work proposes the optimization of the determination of fumaric and maleic acids in NH_3/NH_4Cl 1 mol L^{-1} buffer as supporting electrolyte (Method A), and the determination of butenedioic acid (Method B) using HCl 30 as the supporting electrolyte % (v/v). Measurements were performed by square wave voltammetry using a hanging mercury drop electrode. The results obtained under the optimized conditions, at pH 8.20, presented detection and quantification limits of 0.0689 mg L^{-1} and 0.100 mg L^{-1} for fumaric acid, respectively, and for maleic acid the detection and quantification limits were 0.0108 mg L^{-1} and 0.0209 mg L^{-1}. At pH 1.20, for butenedioic acid, detection and quantification limits of 1.04 mg L^{-1} and 1.32 mg L^{-1} were obtained, respectively. Apple juice samples were purified by solid phase extraction and fortified for voltammetric analysis with fumaric and maleic acid solutions, with recovery values of 99.4% and 98.8%, respectively, at pH 8.20 and 99.5% for butenedioic acid at pH 1.20.
Keywords: fumaric and maleic acids, square wave voltammetry, optimization, apple juice.

I. INTRODUCTION

Butenedioic acid is an unsaturated organic acid with two carboxylic groups and it has geometric isomerism. The structures of the isomers affect the physicochemical properties; fumaric acid has a greater number of intermolecular bonds, while maleic acid has greater facility for making intramolecular hydrogen bonds.

Fumaric acid is used as an additive in foodstuffs and as a raw material for pharmaceutical products and synthetic resins, maleic acid is toxic damaging the respiratory system and the urinary tract (Gulaboski et al., 2001).

The grapes used for wine production, in many regions of the world, have a little amount of acid to produce high quality wines. Thus, it becomes necessary, after alcoholic fermentation with yeasts, the addition of acidulating agents such as fumaric, tartaric, citric, succinic or lactic acid. The use of fumaric acid as an acidulating agent is of paramount importance due to its biological stability, its low cost and due to its inhibitory effect on malolactic fermentation (Pilone et al., 1973).

Fumaric acid is not considered a natural constituent of apple juice prepared without heat treatment; it appears in apple juice due to dehydration of malic acid during the evaporation and / or pasteurization steps. The increase in the content of fumaric acid in apple juice may occur due to adulteration by adding synthetic malic acid that contains fumaric acid as the least contaminant, by overheating the product, by contamination of the juice by microorganisms, or by the processing of apples. damaged or of poor quality (Gokmen and Acar, 2004; Acar et al., 1999; Engel et al., 2008).

Levels of fumaric acid above trace amounts are inconsistent with pure apple juice; therefore the measurement of this organic acid can be used as a check for authenticity of the juice (Gokmen and Acar, 2004).

In apple juice samples, fumaric acid is determined by high performance liquid chromatography (Kadakal and Nas, 2002). Another technique is voltammetric. In this technique, fumaric and maleic acids can be reduced electrochemically to succinic acid (Tarasova et al., 2003; Kvaratskheliya and Kvaratskheliya, 2000).

Square wave voltammetry is considered a versatile, dynamic and sensitive electroanalytical technique. The main advantages of the technique in comparison with the other analytical techniques are its sensitivity, high speed, minimum pre-treatment of the sample, satisfactory selectivity and low cost of instrumentation and maintenance (Economou et al. 2002; Souza et al., 2003).

To optimize these analytical methodologies, experimental designs are used, which are based on statistical principles, making it possible to extract from the system under study the maximum of useful information by carrying out a minimum number of experiments (Neto et al., 2002).

In order to carry out a factorial design, the best model must first be selected. Every process begins with a series of experiments in order to define the qualitative and / or quantitative variables and the levels that are most important. The results must be analyzed and if necessary some modifications must be made. After selecting the variables of greatest significance, the time and cost for carrying out this experiment must be taken into account, that is, evaluating the experimental methodology. In this way, the unselected variables must be fixed throughout the experiment. Normally, a design with two levels, a maximum of three, is used, because design with a greater number of levels become unfeasible (Calado and Montgomery, 2003).

To complete the optimization of the experiment, the significant factors selected, after screening experiments, are used in the response surface methodology. The response surface should be used when the response variables are influenced by many independent variables in order to optimize these responses (Calado and Montgomery, 2003).

The response surface methodology consists of two stages, modeling and displacement, which are repeated as long as necessary to achieve an optimum region of the investigated surface. Modeling is done by adjusting linear or quadratic models to responses obtained after factorial design. The displacement occurs along the maximum inclination path of a given model (Neto et al., 2002).

Thus, the objective of this work was to optimize the analytical methodology through square wave voltammetry to determine butanedioic acid and its isomers, fumaric and maleic acids, in apple juice.

II.   MATERIALS AND METHODS

Cleaning the material and preparing the solutions

The cleaning of the glass material and the polyethylene containers was done with HNO_3 6.0 mol L^{-1} solution in which the glassware was left for a period of 72 hours and then the material was washed with deionized water.

The chemical reagents used were of analytical grade and were not previously subjected to purification.

Two support electrolytes were prepared. To prepare the first, an NH_3 / NH_4Cl buffer at pH 8.2, in two concentrations (1.0 and 2.0 mol L^{-1}), was used ammonium hydroxide (Merck®), hydrochloric acid (Merck ®) and ammonium chloride (Merck®). To prepare the second, HCl 30%, hydrochloric acid (Merck®) was used. Standard solutions of fumaric acid (FLUKA Analytical®) and maleic acid (Merck®) were prepared weekly.

Voltammetric measurements

Metrohm® 797 VA Computrace Voltammetric Analyzer was used for voltammetric measurements, using a drop mercury electrode (HMDE), a silver / silver chloride electrode (Ag/AgCl; KCl 3.0 mol L^{-1}) and a platinum electrode, respectively as working, reference and auxiliary electrodes.

The pH control of the solutions in the voltammetric cell was done with an Orion-Jundilab pH meter, model 720 A, with combined glass electrodes and silver/silver chloride reference (Ag/AgCl; KCl 3.0 mol L^{-1}), with double junction.

To study the effect of pH on voltammetric waves and on both optimization methods, the procedure was performed by adding 11.0 ml of deionized water and 1.0 ml of the supporting electrolyte to the voltammetric cell. The solutions were subjected to the deoxygenation process with ultrapure N_2 for 5 minutes, and then the cathodic scan from -1.00 to -1.85 V was performed with the square wave voltammetric technique.

Then 100 μL of the solution was added to 1.0 g L^{-1} of fumaric acid and 50 μL of the solution to 1.0 g L^{-1} of maleic acid, a new deoxygenation was performed for 3 minutes before a new cathodic scan was performed.

The voltammetric parameters for the study of the potential shift as a function of pH variation were the sweep increment of 15 mV, the amplitude of 50 mV, the frequency of 55 Hz and the drop area of 0.7 mm^2. In the pH 8.2 support electrolyte, maleic and fumaric acids were individually quantified, considering that voltammetric waves occur at different potentials. Using HCl 30% as a supporting electrolyte, fumaric and maleic acids were jointly determined as butenedioic acid because they suffered a reduction in the same potential (Elving and Rosenthal, 1954; Warshowsky et al., 1947).

Depending on the results obtained in the pH study, voltammetric scans were performed for the optimization methodology of the two isomers of butenedioic acid at two different pH values, with the first electrolyte (HCl 30% pH 1.3) and the second electrolyte (NH$_3$/NH$_4$Cl buffer at pH 8.2).

The optimization process for the determination of fumaric and maleic acid separately at pH 8.20 was done by varying the factors amplitude (mV), frequency (Hz), voltage increase (mV), electrolyte concentration (mol L^{-1}) and mercury drop size (mm^2) at two levels in a fractional factorial design. For the optimization process of the methodology for determining fumaric and maleic acid together in 30% HCl, the same factors were varied, except for the concentration of the supporting electrolyte, in a fractional factorial design.

The voltammetric instrument was programmed to perform three scans in each experiment, the average voltammogram was recorded. The tests in the factorial designs were carried out in triplicate and the results of maximum current were recorded to study the levels of the factors to be used later on the response surface.

Sample preparation and analysis

The apple juice samples were centrifuged in an Eppendorf centrifuge, (centrifuge series 5805R) at a speed of 10,000 rpm for 15 minutes and then filtered through a 0.45 μm milipore filter and subjected to the solid phase extraction process (EFS) using C18 cartridges.

In the EFS process, the cartridge was conditioned with successive passages of 10 ml of methanol and 10 ml of water followed by 10 ml of 10% ethanol. After conditioning the C18 cartridge, the samples were eluted, collected and stored in polyethylene bottles in a freezer, for later analysis by voltammetry and HPLC.

For the determinations, after the optimizations, the readings of the blank were made, containing 11.0 mL of deionized water and 1.0 mL of the respective support electrolyte depending on the method. Subsequently, 50 μL of sample was added to the voltammetric cell, in sequence three 50 μL additions of each of the standard solutions of fumaric and maleic acids were made and the voltammograms were recorded after each addition, with the results of maximum average current recorded.

High performance liquid chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was used as a reference method for the quantification of fumaric and maleic acids. The analysis was performed on a Shimadzu HPLC, model SPD-10A VA coupled to a UV detector at a wavelength of 210 nm.

The column used was the SCR-101H of 30 cm x 7.9 mm in diameter, using a flow of the mobile phase in the column of 0.6 mL min^{-1}, the pressure used in the column was 24 Kgf and the mobile phase was acid phosphoric 1% v/v in water. The volume of sample injected was 20 μL

III.   RESULTS AND DISCUSSION

The development of this methodology is based on the possibility of determining the two isomers of butenedioic acid at different pH values, in view of the displacement of the potential as a function of pH, as shown in Figure 1.

Table 1: pKa values for butenedioic acid isomers

	pKa$_1$	pKa$_2$
Fumaric acid	3.45	4.49
Maleic acid	1.83	6.07

(Gulaboski et al., 2001)

At pH 1.30, a single peak was observed referring to the reduction of fumaric acid and maleic acid to succinic acid, in the approximate potential of -0.60 V. In this pH value, the two acids are protonated, according to the pKa$_1$ and pKa$_2$ values of each acid shown in table 1. In this way, the two isomers can be determined simultaneously.

On the other hand, at pH 8.15 the two acids are completely deprotonated and the voltammetric waves are visualized separately for the maleic acid at the potential of -1.38 V and for the fumaric acid at the potential of -1.72 V.
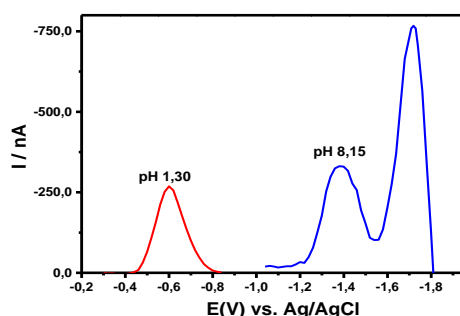
Figure 1: Voltamograms of the mixture of fumaric and
maleic acids as a function of pH in the two support electrolytes.

Method A
Factor screening
A fractional factorial design was developed to investigate the relative importance of the factors, scan increment (SI), amplitude (AM), frequency (FR), concentration of the support electrolyte (C) and drop area (DA), in the voltammetric response.

The levels chosen for each factor are listed in table 2 and the factorial design with the responses in each trial in table 3.

Table 2: Factors and levels chosen for fractional factorial design 2_V^{5-1}.

Symbol	Factors	Levels	
		-	+
SI	Scan Incremente (mV)	10	20
AM	Amplitude (mV)	18	70
FR	Frequency (Hz)	20	90
C	Electrolyte concentration (mol L^{-1})	1.0	2.0
DA	Drop Area (mm^2)	0.2	0.7

Table 4 shows that the values of the contrasts vary from 9.86 nA to 257.77 nA, so it is reasonable to consider that the contrasts l1, l2, l3 and l5 that present values above 100 nA as being those that have greater relative importance, therefore, should be analyzed.

The contrast l1 represents the scanning increment (SI), l2 the amplitude (AM), l3 the frequency (FR) and l5 the drop area (DA) and all these contrasts are of relative importance due to the current values presented.

The l5 contrast value suggests that an increase in the drop area produces better responses. However, the increase in the area of the mercury drop produced irregular voltamograms, probably because the large surface area formed impairs the polarization of the drop, causing an increase in the capacitive current value. Therefore, the drop area was fixed at 0.7 mm2.

Table 3: Fractional factorial design and respective responses.

Experiment	Factors					Response (nA)
	SI	AM	FR	C	DA	
01	-	-	-	-	+	67.40
02	+	-	-	-	-	40.08
03	-	+	-	-	-	120.7
04	+	+	-	-	+	663.8
05	-	-	+	-	-	79.15
06	+	-	+	-	+	293.7
07	-	+	+	-	+	605.8
08	+	+	+	-	-	621.3
09	-	-	-	+	-	28.94
10	+	-	-	+	+	147.6

11	-	+	-	+	+	181.7
12	+	+	-	+	-	198.3
13	-	-	+	+	+	257.4
14	+	-	+	+	-	106.6
15	-	+	+	+	-	185.7
16	+	+	+	+	+	686.9

As the contrast l4, which represents the concentration of the electrolyte, has a low current value, it has no relative importance and, therefore, any concentration value within the studied concentration range can be used. Thus, it was decided to work with the lowest concentration of the support electrolyte of the 1.0 mol L^{-1}.

After the analysis of fractional design, a reduction in the number of factors to be evaluated was obtained and, therefore, new values were chosen for the levels of the three factors of greatest relative importance, SI, AM and FR, as can be seen in the complete factorial design (2^3), in table 5.

Table 4: Estimates of fractional factorial contrasts and their relationship to the effects of the complete factorial.

Relationship between columns	Contrasts		Response (nA)*
1 = 2345	$l_1 = l_{2345}$	$l_1 = 1 + 2345$	**131.28**
2 = 1345	$l_2 = l_{1345}$	$l_2 = 2 + 1345$	**257.77**
3 = 1245	$l_3 = l_{1245}$	$l_3 = 3 + 1245$	**150.87**
4 = 1235	$l_4 = l_{1235}$	$l_4 = 4 + 1235$	-64.70
5 = 1234	$l_5 = l_{1234}$	$l_5 = 5 + 1234$	**213.10**
12 = 345	$l_{12} = l_{345}$	$l_{12} = 12 + 345$	92.52
13 = 245	$l_{13} = l_{245}$	$l_{13} = 13 + 245$	-31.46
14 = 235	$l_{14} = l_{235}$	$l_{14} = 14 + 235$	-9.86
15 = 234	$l_{15} = l_{234}$	$l_{15} = 15 + 234$	38.63
23 = 145	$l_{23} = l_{145}$	$l_{23} = 23 + 145$	37.66
24 = 135	$l_{24} = l_{135}$	$l_{24} = 24 + 135$	-79.75
25 = 134	$l_{25} = l_{134}$	$l_{25} = 25 + 134$	85.25
34 = 125	$l_{34} = l_{125}$	$l_{34} = 34 + 125$	19.15
35 = 124	$l_{35} = l_{124}$	$l_{35} = 35 + 124$	45.00
45 = 123	$l_{23} = l_{123}$	$l_{45} = 45 + 123$	-24.55
I = 12345	$l_I = $ média+1/2(12345)		**256.49**

(*) Contrasts in bold are the most important.

Table 5: Factors and levels chosen for complete factorial design 2^3.

Symbol	Factors	Levels	
		-	+
SI	Scan Increment (mV)	10	20
AM	Amplitude (mV)	30	110
FR	Frequency (Hz)	35	110

Table 6: Complete factorial design.

Experiment	Factors			Response (nA)
	SI	AM	FR	
01	-	-	-	299.90
02	+	-	-	478.98
03	-	+	-	387.09
04	+	+	-	498.56
05	-	-	+	456.90
06	+	-	+	467.90
07	-	+	+	867.00

08	+	+	+	899.05

The response related to the complete factorial design are shown in table 6. It can be seen in table 7 that all the main effects SI, AM and FR and the interactions AM x FR and FR x SI were significant by the t test and $\alpha = 0.05$.

Table 7: Estimates of the main and interaction effects for the full factorial.

Estimation of effects ± error (*)	
Average	**527.86 ± 6.19**
Main effects	
SI	**85.87 ± 12.39**
AM	**232.57 ± 12.39**
FR	**249.86 ± 12.39**
2nd. order interaction	
SIxAM	-7.56 ± 12.39
SIxFR	**-56.60 ± 12.39**
AMxFR	**186.32 ± 12.39**

(*) Estimates of the effects in bold are the most important relative.

Response Surface

To study the three most significant factors: scan increment (SI), amplitude (AM) and frequency (FR), central composite design (CCD) was used.

Table 8 shows the central composite design (with $\alpha = 1.682$) built for the three factors under the conditions described and the responses.

The calculations referring to the values of the coefficients and the error of each factor studied on the response surface are listed in table 9.

Table 8: Result of central composite design (CCD) design with k = 3.

Ensaio	SI (X_1)	AM (X_2)	FR (X_3)	SI (mV)	AM (mV)	FR (Hz)	I(nA)
01	-1	-1	-1	10.00	30.00	35.00	290.40
02	1	-1	-1	20.00	30.00	35.00	449.95
03	-1	1	-1	10.00	110.0	35.00	349.41
04	1	1	-1	20.00	110.0	35.00	469.16
05	-1	-1	1	10.00	30.00	110.0	412.26
06	1	-1	1	20.00	30.00	110.0	436.34
07	-1	1	1	10.00	110.0	110.0	816.48
08	1	1	1	20.00	110.0	110.0	866.43
09	-1.682	0	0	6.590	70.00	72.50	465.89
10	1.682	0	0	23.41	70.00	72.50	581.78
11	0	-1.682	0	15.00	2.720	72.50	20.110
12	0	1.682	0	15.00	137.3	72.50	448.34
13	0	0	-1.682	15.00	70.00	9.430	864.41
14	0	0	1.682	15.00	70.00	135.6	778.13
15	0	0	0	15.00	70.00	72.50	703.44
16	0	0	0	15.00	70.00	72.50	604.33
17	0	0	0	15.00	70.00	72.50	693.14
18	0	0	0	15.00	70.00	72.50	663.30

Table 9: Result of central composite design (CCD) design with k = 3.

Symbol	Coefficients (*)
Average	**666.37 ± 22.23**
Main effects	
SI	**40.141 ± 12.05**
AM	**-51.721 ± 12.05**
FR	**119.55 ± 12.05**
2nd. order interaction	
SIxSI	**-154.09 ± 12.52**
AMxAM	**60.584 ± 12.52**
FRxFR	**53.413 ± 12.52**
SIxAM	-1.741 ± 15.74
SIxFR	-25.659 ± 15.74
AMxFR	**94.511 ± 15.74**

(*) Estimates of the effects in bold are the most important relative.

The model equation (1) adjusted for the significant coefficients and their respective errors is:

$$i_P = 666.37_{\pm 22.23} + 40.14_{\pm 12.05} (SI) - 51.72_{\pm 12.05} (AM) + 119.55_{\pm 12.05} (FR) - 154.09_{\pm 12.52} (SI)^2 + 60.58_{\pm 12.52} (AM)^2 + 53.41_{\pm 12.52} (FR)^2 + 94.51_{\pm 15.74} (AMxFR) \qquad (1)$$

To obtain the response surface, the SI values were varied from -1.682 to 1.682 and the best current response obtained was at the (+) level that has the value of the coded variable equal to 1.0 and the value of the decoded variable equal to 20 mV. In this way, the SI value was fixed at 20 mV and the surface was obtained between the main factors AM and FR.
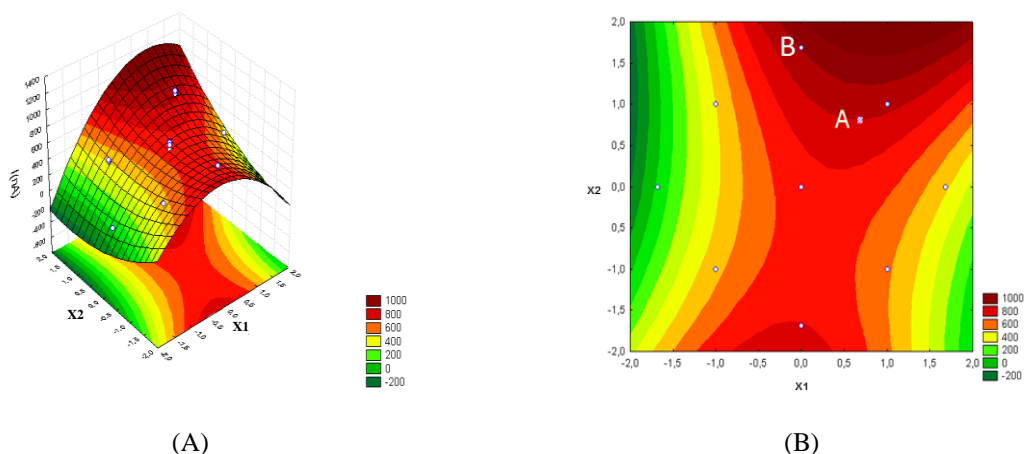

(A)   (B)

Figure 2: (A) Response surface with the scan increment variable set at level (1), 20 mV.
X1 = AM (mV), X2 = FR (Hz) in terms of coded variables. (B) Projection of the response surface.

In figure 2, it is possible to observe a surface that presents two regions of maximum represented by the color red with a current value close to 1000 nA, this can be better visualized in figure 2 (B), which is the projection of the response surface.

The maximum point would be at the point represented by the coded variables   X1 = 0 and X2 = 1.682 (Point B), however the voltammetric wave obtained was distorted, causing a loss of symmetry. Due to the distortion of the voltammetric wave at the maximum point, the values of X1 = 0.62 and X2 = 0.72 (Point A) were chosen and used later in all measurements.

The results obtained on the response surface indicate that by increasing the amplitude and frequency values, the current response increases proportionally. However, the increase in frequency causes a shift in the reduction potential to more negative values in the voltammetric peaks of fumaric acid and maleic acid, impairing the resolution.

The optimized parameters are shown in table 10, which show the working conditions in which the sample analysis experiments were carried out.

Table 10: Parameters optimized for the analysis of fumaric acid and square wave maleic acid, using the maleic acid current.

Parameters	Optimal values	Estimated current
Initial potential	-1.20 V	783.18 nA
Final potential	-1.90 V	
Scan increment	20.00 mV	
Amplitude	94.8mV	Observed current
Frequency	99.5 Hz	786.19 nA
Scan speed	1.989 mVs^{-1}	
Drop area	0.70 mm^2	Relative error: +0.38 %

Figure 3 shows the voltammograms of maleic and fumaric acids, with the respective analytical curves shown in Figure 4. The peak potentials for maleic and fumaric acids using the optimized parameters were equal to -1.38 Volts and - 1.72 Volts, respectively.
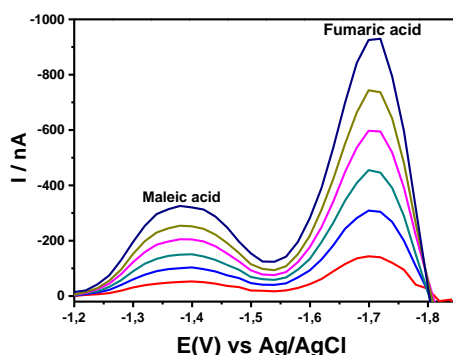

Figure 3: Voltamograms referring to the standards for making the analytical curves to assess the linearity of the responses of maleic and fumaric acids.
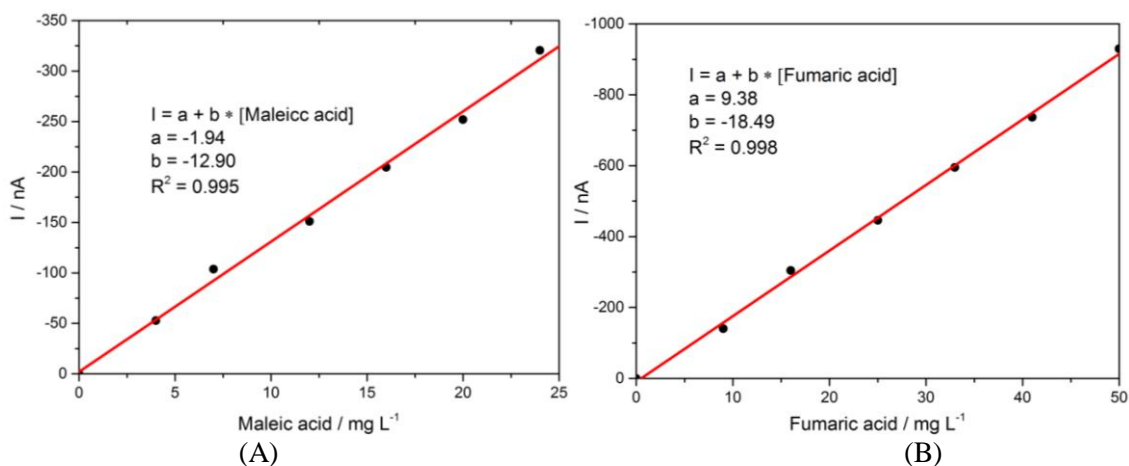

Figure 4: Analytical curves of Maleic (A) and Fumaric (B) acids.

The detection limit of 0.011 mg L^{-1} and a quantification limit of 0.021 mg L^{-1} for maleic acid were estimated. For fumaric acid, the detection limit was 0.069 mg L^{-1} and the quantification limit was 0.340 mg L^{-1}.

Method B
Factor screening

A fractional factorial design 2_{IV}^{4-1} was developed to investigate the relative importance of the factors, scan increment (SI), amplitude (AM), frequency (FR) and drop area (DA), in the voltammetric response. The levels chosen for each factor are listed in table 11 and the factorial design with the responses in each trial in table 12.

Table 11: Factors and levels chosen for fractional factorial design 2^{4-1}_{IV}.

Symbol	Factors	Levels	
		-	+
SI	Scan increment (mV)	6	18
AM	Amplitude (mV)	15	55
FR	Frequency (Hz)	20	80
DA	Drop area (mm^2)	0.20	0.70

Tabela 12: Table 12. Fractional factorial design and respective responses.

Experiment	Factors				Response (nA)
	SI	AM	FR	DA	
01	-	-	-	-	161.45
02	+	-	-	+	359.71
03	-	+	-	+	505.01
04	+	+	-	-	417.86
05	-	-	+	+	553.54
06	+	-	+	-	444.67
07	-	+	+	-	535.02
08	+	+	+	+	1799.0

In table 13 it can be seen that the values of the contrasts vary from 233.52 nA to 597.03 nA, therefore it is reasonable to consider those contrasts with values above 300 nA as being those that have the greatest relative importance and therefore the contrasts ℓ1, ℓ2 ℓ3 and ℓ4 should be analyzed.

The contrasts ℓ1, ℓ2, ℓ3 and ℓ4 represent SI, AM, FR and DA respectively; all of these contrasts are of relative importance due to the current values presented. The contrast value ℓ4 suggests the use of the level (+) of the variable DA, that is 0.7 mm^2, so the drop area was fixed at this value.

Table 13: Estimates of fractional factorial contrasts 2^{4-1}_{IV} and their relationship to the effects of the complete factorial.

Relationship between columns		Contrasts	Response (nA)*
1 = 234	$\ell_1 = \ell_{234}$	$\ell_1 = 1 + 234$	**316.56**
2 = 134	$\ell_2 = \ell_{134}$	$\ell_2 = 2 + 134$	**434.38**
3 = 124	$\ell_3 = \ell_{124}$	$\ell_3 = 3 + 124$	**472.05**
4 = 123	$\ell_4 = \ell_{123}$	$\ell_4 = 4 + 123$	**414.57**
12 = 34	$\ell_{12} = \ell_{34}$	$\ell_{12} = 12 + 34$	271.86
13 = 24	$\ell_{13} = \ell_{24}$	$\ell_{13} = 13 + 24$	261.00
14 = 23	$\ell_{14} = \ell_{23}$	$\ell_{14} = 14 + 23$	233.52
I = 1234	ℓ_I=média + 1/2(1234)		**597.03**

(*) Estimates of the contrasts in bold were significant by the t test.

In the analysis of the voltammograms obtained in each test, a loss of symmetry and displacement of the voltammetric wave was observed, probably because of the high value of the factors in the levels (+). In this way, it was decided to decrease the studied range, reducing the values of the frequency and the sweep increment. In the new design, a complete factorial 2^3, the drop area was kept at 0.7 mm^2 and the other factors with their levels according to table 14.

Table 14: Factors and levels chosen for the complete factorial design 2^3.

Symbol	Factors	Levels	
		-	+
SI	Scan increment (mV)	3	7
AM	Amplitude (mV)	35	60
FR	Frequency (Hz)	45	65

The complete factorial design carried out presented the current responses according to table 15.

Table 15: Complete factorial design and current response.

Experiment	Factors			Response (nA)
	SI	AM	FR	
01	-	-	-	623.00
02	+	-	-	798.00
03	-	+	-	700.00
04	+	+	-	924.89
05	-	-	+	681.90
06	+	-	+	987.09
07	-	+	+	934.87
08	+	+	+	1088.0

Table 16: Estimation of the main and interaction effects for the full factorial design.

Estimation of effects ± error (*)	
Average	**831.68 ± 5.14**
Main Effects	
SI	**202.76 ± 10.27**
AM	**139.65 ± 10.27**
FR	**151.27 ± 10.27**
2nd. order interactions	
SIxAM	-23.25 ± 10.27
SIxFR	3.25 ± 10.27
AMxFR	**32.83 ± 10.27**
3rd. order interaction	
SIxAMxFR	**-50.87 ± 10.27**

(*) Estimates in bold are the most important.

The main effects SI, AM and FR and the second order interaction AM and FR were significant by the t test, $\alpha = 0.05$, as shown in figure 16.

Response surface

After screening the factors and carrying out the complete factorial design, the three most significant factors SI, AM and FR, were used in the central composite design (CCD), as shown in table 17.

The coefficients of each factor and their respective error values are found in table 18, where it can be seen that the three main factors and the mean are significant by the t test.

Equation (2) obtained in the regression takes into account only the mean, the factors SI, AM and FR and the interactions, which were significant.

$$Z = 848.80_{\pm 3.31} + 110.31_{\pm 1.79} (SI) - 78.57_{\pm 1.79} (AM) + 71.25_{\pm 1.79} (FR) - 15.29_{\pm 1.86} (SI)^2 - 13.77_{\pm 1.86} (AM)^2 - 12.08_{\pm 2.34} (SIxAM) + 12.08_{\pm 2.34} (AMxFR) \quad (2)$$

Table 17: Result of central composite design (CCD) design with k = 3.

Test	SI (X_1)	AM (X_2)	FR (X_3)	SI (mV)	AM (mV)	FR (Hz)	I (nA)
01	-1	-1	-1	3.00	35.0	45.0	610.27
02	1	-1	-1	7.00	35.0	45.0	779.53
03	-1	1	-1	3.00	60.0	45.0	693.77
04	1	1	-1	7.00	60.0	45.0	923.04
05	-1	-1	1	3.00	35.0	65.0	682.09
06	1	-1	1	7.00	35.0	65.0	940.27
07	-1	1	1	3.00	60.0	65.0	923.04

08	1	1	1	7.00	60.0	65.0	1023.0
09	-1,682	0	0	1.64	47.5	55.0	581.11
10	1.682	0	0	8.36	47.5	55.0	1027.0
11	0	-1.682	0	5.00	26.5	55.0	653.03
12	0	1.682	0	5.00	68.5	55.0	963.67
13	0	0	-1.682	5.00	47.5	38.2	728.07
14	0	0	1.682	5.00	47.5	71.8	972.61
15	0	0	0	5.00	47.5	55.0	856.94
16	0	0	0	5.00	47.5	55.0	840.72
17	0	0	0	5.00	47.5	55.0	849.15
18	0	0	0	5.00	47.5	55.0	848.89

To obtain the response surface, the FR (frequency) values were varied from -1.682 to 1.682 and the best current response obtained was at the level (0) whose decoded variable represented 55 Hz. Thus, this variable was fixed at this value and the surface obtained between the main factors SI and AM.

Figure 5 (A) shows the response surface obtained, with the points distributed over this surface. A better visualization of this surface can be seen in figure 5 (B), which shows a projection of this surface. The voltammogram obtained by the mathematical maximum data was distorted and with a displacement from potential to more negative potential. Therefore, point B (X1 = 1; X2 = 0.5) was chosen because it presents a better symmetry of the voltammetric peak resulting from the reduction of butenedioic acid.

Table 18: Coefficients for the response surface model.

Symbol	Coefficients (*)
Average	**848.80 ± 3.31**
Main effects	
SI	**110.31 ± 1.79**
AM	**78.57 ± 1.79**
FR	**71.25 ± 1.79**
2nd order interactions	
SIxSI	**-15.29 ± 1.86**
AMxAM	**-13.77 ± 1.86**
FRxFR	1.07 ± 1.86
SIxAM	**-12.28 ± 2.34**
SIxFR	-5.05 ± 2.34
AMxFR	**12.08 ± 2.34**

(*) Coefficients in bold were significant by the t test.

Figure 5: (A) Response surface with the Frequency (FR) variable set at the level (0), 55 Hz. X1 = SI (mV), X2 = AM (mV) in terms of coded variables. (B) Projection of the response surface.


(A)

(B)
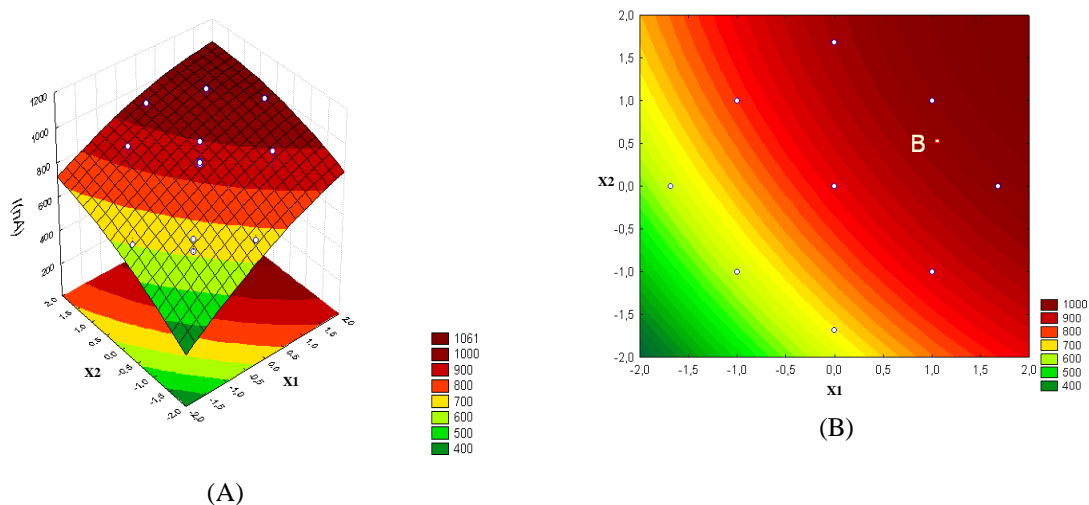
The values of the parameters optimized for analyzing butenedioic acid are shown in table 19.

Table 19: Optimized parameters for the analysis of butenedioic acid.

Optimized parameters	Optimal values	Estimated current*
Initial potential	-0.30 V	998.40 nA
Final potential	-0.75 V	
Scan increment	7.00 mV	
Amplitude	53.75 mV	Observed current*
Frequency	55.00 Hz	937.85 nA
Scan speed	0.386 mV s^{-1}	
Drop area	0.700 mm^2	*Erro relativo: -6.07 %

Figure 6 shows the square wave voltamograms of butenedioic acid and the analytical curve. The limit of detection equal to 1.04 mg L^{-1} and the limit of quantification equal to 1.32 mg L^{-1} were estimated for butenedioic acid.

Figure 6: (A) Voltamograms referring to the standards for evaluating the working range of butenedioic acid; (B) Analytical curve of butenedioic acid.

(A)

$I = a + b * [\text{Butenodioic acid}]$
a = 0.0662
b = -0.0282
R^2 = 0.996

(B)
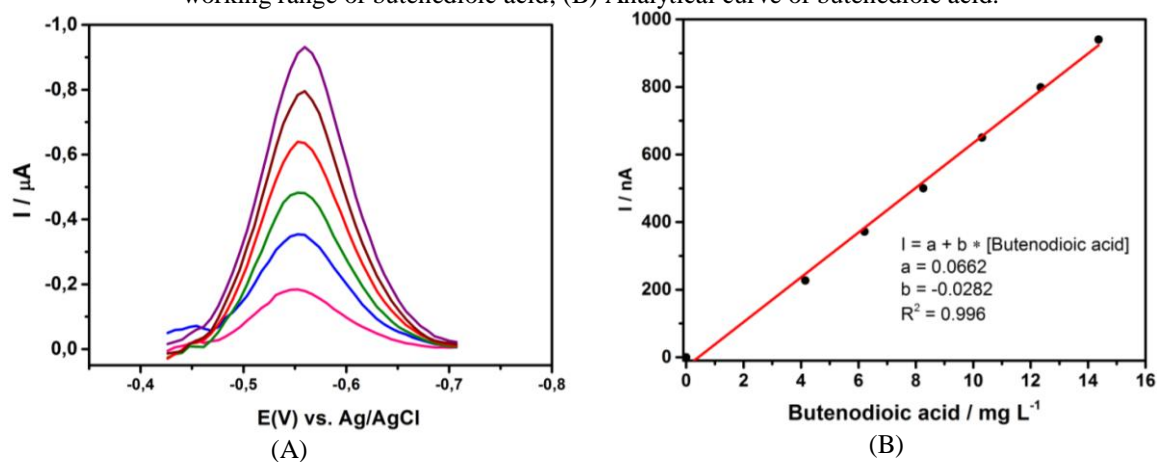
Application of optimized methodologies to apple juice
Figure 7 shows the standard additions for the determination of fumaric and maleic acids in sample 4 of apple juice, by both methods.

Figure 7: Voltamograms of sample 4 of apple juice analyzed using the optimized parameters of Method A and B, by adding standards.

(A)   (B)
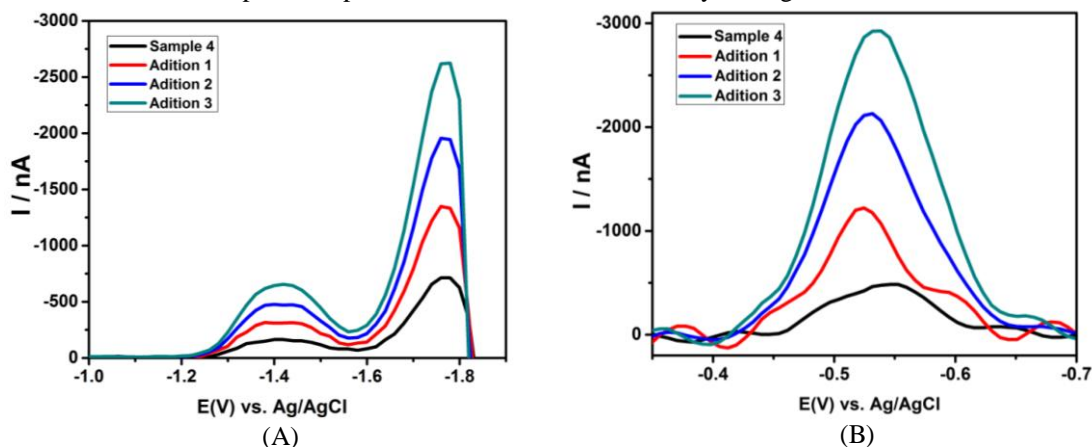
The comparison method used to validate the two methodologies was HPLC, as can be seen for the two isomers for method A in table 20 and for butenedioic acid by method B in table 21.

Table 20: Comparison between the values of fumaric acid and maleic acid in mg L^{-1}, in apple juice, by voltammetric method A and by HPLC.

Sample	Method A		HPLC	
	Fumaric acid	Maleic acid	Fumaric acid	Maleic acid
01	4.68 ± 0.03	1.22 ± 0.01	4.66 ± 0.04	1.24 ± 0.02
02	5.83 ± 0.02	0.27 ± 0.02	5.92 ± 0.01	0.28 ± 0.04
03	3.40 ± 0.02	0.19 ± 0.01	3.37 ± 0.03	0.20 ± 0.02
04	8.27 ± 0.11	1.40 ± 0.01	8.31 ± 0.15	1.41 ± 0.02
05	5.01 ± 0.02	1.13 ± 0.01	5.10 ± 0.03	1.14 ± 0.01
06	4.76 ± 0.03	0.62 ± 0.02	4.80 ± 0.04	0.64 ± 0.01

Table 21: Comparison between the concentrations of butenedioic acid in mg L^{-1}, in apple juice, by voltammetric method B and by HPLC.

Sample	Method B	HPLC
01	5.77 ± 0.01	5.90 ± 0.01
02	6.13 ± 0.02	6.20 ± 0.01
03	3.65 ± 0.01	3.57 ± 0.03
04	9.73 ± 0.13	9.72 ± 0.02
05	6.20 ± 0.03	6.24 ± 0.02
06	5.43 ± 0.01	5.44 ± 0.03

IV.   CONCLUSION
The process of optimizing the methodologies for the individual determination of isomers of butenedioic acid (fumaric and maleic acids) and together as butenedioic acid, using the square wave voltammetry technique, proved to be simple, fast and easy to perform. The methods used were shown to be accurate and reproducible, not requiring the pre-concentration stage of the samples, the stage of extraction in solid phase is simple and necessary due to the use of complex samples, such as apple juice.

The most significant parameters were the increase in voltage, amplitude and frequency, using an appropriate support electrolyte in each method. After optimization, the methods were properly validated and the results obtained for each sample demonstrate the compatibility of the two methods, with the levels found of butenedioic acid in the samples equivalent to the sum of the content of fumaric acid and maleic acid.

At pH 8.20, the concentrations of each isomer were determined separately and at pH 1.30, the concentration of butenedioic acid was determined, so that one method complemented the other, endorsed by HPLC.

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