A comparative study on the determination of lactic acid in silage juice by colorimetric, high-performance liquid chromatography and enzymatic methods

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Abstract: The determination of lactic acid in the silage juice of artichokes with different additives (formid acid, molasses and NaCl) by the colorimetric method and its comparision with the high-performance liquid chromatography and enzymatic methods was investigated. The lactic acid content of the artichoke with molasses (62.1 g kg^{-1}) was higher than that of those with formic acid, or NaCl and without any additive (39.3, 33.0 and 43.2 g kg^{-1}, respectively). However, this effect was not significant ($P > 0.05$). There were significant differences on the method of measuring lactic acid of the artichoke silages ($P < 0.001$). The use of the enzymatic method resulted in a higher (75.6 g kg^{-1}) lactic acid content than when the colorimetric or HPLC methods were employed (with results of 42.0 and 28.9 g kg^{-1}, respectively). However, the levels of lactic acid in silage juices found using the colorimetric and HPLC methods were not different, and recovery percentages, by using the colorimetric method, were satisfactory (103.78%), when the detection limit at maximum level (30 μg ml^{-1}) was not exceeded.

Keywords: colorimetric; HPLC; enzymatic; lactic acid; silage

INTRODUCTION

Ensilage is a method of preserving crops by natural fermentation in anaerobic conditions.[1] The major products of anaerobic bacterial fermentation are ethanol, volatile fatty acids (C_2 to C_6) and succinic and mainly lactic acids.[2] Lactic acid is the best indicator of the fermentative value of silage: it produces a decrease in pH to a level at which the undesirable fermentations that produce butyric acid and degrade amino acids are inhibited.

In addition, in several other biological materials, such as fruit, vegetable juices, wine, yoghourt, cheese, meat products, etc, the determination of lactic acid is used as an indicator of the fermentative processes that take place in their preparation, storage and preservation.[3,4]

The determination of lactic acid has been carried out by high-performance liquid chromatography (HPLC),[5] gas chromatography (GC),[6] colorimetry[7] and enzymatic methods.[8] The most common method used in agricultural sciences is the colorimetric lactic acid assay for silage and ruminal samples, as this method does not require any special instrumentation or preparation and is simple, reliable and sensitive. However, it does present some problems in terms of erratic responses in the standard curves due to an unstable dissolution of the chromagen. Taylor[9] modified the colorimetric procedure and avoided the traditional problems, using standard solutions.

The aim of this study was to determine the lactic acid in silage juices by the colorimetric method and to compare it with the high-performance liquid chromatography (HPLC) and enzymatic procedures.

EXPERIMENTAL

Sample preparation

The study materials consisted of artichoke (*Cynara scolymus* L) the by-product from the industrial processing of artichoke hearts. The artichokes were washed and scalded at 90 °C for 20 min, and the hearts removed mechanically. The outer bracts and stems are the principal parts of this by-product.

The material was transferred to the laboratory and ensiled in microsilos with a capacity of 12.50 litres following the procedure described by Megías *et al.*[10] The by-product was treated with three different additives: (1) formic acid at 20% in a dose of 2 ml kg^{-1}

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(2) molasses (50 g kg^{-1}) and (3) sodium chloride (30 g kg^{-1}). A fourth group was maintained the control treatment, and three replicates per treatment were used.

After 100 days ensiling, 20 g of fresh material were crushed and homogenized with 100 ml of deionized water. The sample was macerated at room temperature for 1 h. Silage samples were filtered through four layers of cheesecloth.[10] This resulting filtered liquid was used to determine the amount of lactic acid.

Colorimetric method

The optimized method for lactic acid determination as developed by Taylor[9] is as follow:

Reagents

Concentrated H_2SO_4 (96%), 4% $CuSO_4.5H_2O$ in deionized water, 1.5% *p*-phenylphenol (pPP) in 95% ethanol and lactic acid were used. All reagents were of analytical reagent grade.

Standard method and curve

For the standard curve, standard solutions of 0–30 μg ml^{-1} (in 5-μg increments) were used. Six millilitres of concentrated H_2SO_4 in borosilicate tubes was added to 1 ml of standard solutions and mixed in a vortex mixer. The quantity of acid was defined here as 82% acid. The mixed solutions were incubated at 95–100 °C for 10 min in a steam water bath. The tubes were cooled to room temperature using a water bath and, subsequently, 100 μl $CuSO_4$ reagent and then 200 μl pPP reagent were added, and mixed well using a vortex mixer. The tubes were kept at room temperature (no less than 20 °C) for at least 30 min and the absorbance level recorded at 570 nm. The blanks should show values of 0.2–0.5 compared to water.

Quantification and recovery

After filtering the silage samples, this liquid was diluted 1:100 with deionized water and 1 ml of this diluted filtrate was analysed directly.

The accuracy of the present method was determined by using two filtrates of different silages and standard lactic acid solutions with known concentrations. Water or standard solution A or B (0.5 ml) were added to 0.5 ml of silage juice 1 (formic acid treatment) or silage juice 2 (sodium chloride treatment). Standard concentrations of lactic acid were added, 15 μg (0.5 ml of standard solution of 30 μg ml^{-1} of lactic acid) and 30 μg (0.5 ml of standard solution of 60 μg ml^{-1}) for solution A and B respectively.

HPLC method

The method for the determination of lactic acid in silages was that developed by Megías *et al.*[10]

Instrumentation and chromatography conditions

A Kontron pump HPLC system (Model 422) with Kontron automatic injector (Model 465) was used for the analyses. The integration method used was a Kontron system data set. Separations were achieved by using an Inter Action GC-801 precolumn and an Inter Action ION-300 column (300 mm × 7.8 mm ID). The mobile phase was a solution of 0.01 N H_2SO_4 run at 0.8 ml min^{-1}. The injection volume was 50 μl. A Kontron UV detector (Model 481) was used and analyses were carried out at 210 nm. The running time for the analysis was 30.01 min.

Preparation of standard solutions

Standard solutions of lactic acid were prepared in deionized water at 500, 1000 and 2000 μg ml^{-1}. Citric acid solution (2 g $litre^{-1}$, 0.5 ml) as internal standard was added to 1 ml of each standard solution. The peak-area ratio to citric acid was determined for each standard solution and was plotted against the concentration of lactic acid.

Quantification and recovery

Silage juice samples were not diluted for this method. Instead, they were centrifuged at 15000 rpm for 15 min and filtered through a Millipore 0.45-μm filter (Millipore). To 1 ml of this filtrate 0.5 ml of internal standard solution was added, and analysed directly.

The precision of the HPLC method was determined using a filtrate of silage. Water or standard solutions (0.5 ml) were added to 0.5 ml of prepared 100-days artichoke silage juice. The added standard concentrations of lactic acid were 300, 500, 800 or 1000 μg (0.5 ml of lactic acid standard solutions at 600, 1000, 1600 and 2000 μg ml^{-1}, respectively).

Enzymatic method

Enzymatic standard analysis (Boehringer Mannheim, Methods of Analysis, Cat No 1112 821) for the determination of D(−)- and L(+)-lactic acid in silage juices was used.

Statistical analysis

All the data were subjected to an analysis of the variance (ANOVA)[11] and the differences between the means were tested, using Duncan's New Multiple Range Test.

Data refering to the lactic acid of silage were analysed using ANOVA for a two-way comparison with interactions. The model used was:

$$Y_{ij} = \mu + A_i + B_j + AB_{ij} + \varepsilon$$

where A, B and AB were the effects of the method type, the treatment type and the method type × treatment type interaction, respectively.

The standard curves were determined by fitting a linear function. The relationship between the results obtained using the colorimetric, HPLC and enzymatic methods in silage juices was established by means of a correlation matrix.

	Treatment type					
	Formic acid	*Molasses*	*NaCl*	*None*	*SE*	*Sig*[a]
Colorimetric method	31.2	66.6	31.5	38.7	7.2	NS
HPLC method	30.0	32.9	22.7	30.0	4.1	NS
Enzymatic method						
D(−)+L(+) lactic acid	67.6	105.7	55.6	73.7	11.6	NS
L(+) lactic acid	49.8	102.2	37.1	43.7	9.3	NS
D(−) lactic acid	17.8	3.4	18.4	30.0	5.1	NS
Statistic pooled data						
Methods type (A)[a]	*Treatment type (B)*[a]			*A × B*[a]		*SE*
***	NS			NS		3.6

[a] Significance: *** $P<0.001$; NS $P>0.05$.

Table 1. Lactic acid levels (g kg^{-1} DM) in artichoke silages with different additives, obtained by colorimetric, HPLC and enzymatic methods

RESULTS AND DISCUSSION

Lactic acid in silages

The lactic acid contents of different artichoke silages calculated by different analytical methods are shown in Table 1. The mean values for lactic acid content of artichokes with molasses (62.1 g kg^{-1}) were higher than those of artichokes with formic acid, NaCl or without any additive (39.3, 33.0 and 43.2 g kg^{-1}, respectively). The lactic acid content was higher with the colorimetric method (66.6 g kg^{-1}) than the HPLC method (32.9 g kg^{-1}) with molasses additive. This effect would be due to a reaction of the compounds present in molasses, such as sugars. Taylor[9] reported that some sugars, such as rhamnose, arabinose and fucose, showed some interference absorbance in the assay. The concentrations of lactic acid determined by each assay method varied when the analytical methods were used with natural samples; the results were liable to the vagaries of natural variation. However, this effect was not significant ($P>0.05$).

The analytical methods used significantly affected the lactic acid measurement ($P<0.001$) of the artichoke silages. The enzymatic methods showed higher (75.6 g kg^{-1}) lactic acid content than the colorimetric or HPLC methods (42.0 and 28.9 g kg^{-1}, respectively). This effect could mean that the enzymatic method was accurate as the sum of two stereoisometric forms of lactic acid could increase the risk of error. In addition, King and White[12] determined that the colorimetric assay was more accurate than the enzymatic assay owing to a lack of specificity in lactic dehydrogenase. However, the method type × treatment type interaction was not significant ($P<0.05$).

The lactic acid concentrations with the colorimetric method were 31.2, 66.6, 31.5 and 38.7 g kg^{-1}, for artichoke silages with formic acid, molasses, NaCl or without any additive. Similar results were found by Megías *et al*[13] for the same by-product silage.

Colorimetric and HPLC methods

In the present study, the traditional problems of the colorimetric assay, such as precipitates and unstable dissolution of the pPP reagent, were not found. In this method, acetaldehyde is released from lactic acid by hot H_2SO_4 at 95–100 °C. The acetaldehyde reacts with copper and pPP to yield a blue or blue-purple chromagen. In the typical assay, the concentration of sulfuric acid is defined as 79% acid. However, Taylor[9] reported that the maximum absorbance for colorimetric lactic determination is got at 82% of acid, refers to the amount of concentrated sulfuric acid in the mixture.

The relationship between the standard concentrations of lactic acid and absorbance at 570 nm was linear ($P<0.001$) over a wide range of 0–30 μg ml^{-1} ($y=-0.89+9.35x$, $R^2=0.9920$), and with a detection limit at maximum level of 30 μg ml^{-1}.

The accuracy of the colorimetric method for silage juices was expressed as percentage recovery (Table 2). The recovery percentages were satisfactory for two of the silages juices (mean of 103.78%) when the added standard concentration of lactic acid was 15 μg. This recovery percentage was similar to that of high-performance analytical assays.[14,15] By contrast, the recovery percentages were low for silage juices (mean of 89.30%) with the addition was of 30 μg of lactic acid, because the maximum level of the detection method was exceeded by 20–30%.

High-performance liquid chromatography (HPLC) methods have been developed for the quantification of

	Juice[a]	*Juice2*[a]
Lactic acid (μg)	5.79	8.81
Solution A added (μg)	15.00	15.00
Solution A + juice (μg)	22.75	23.37
Recovery (%)	*109.42*	*98.15*
Solution B added (μg)	30.00	30.00
Solution B + juice (μg)	33.30	33.21
Recovery (%)	*93.04*	*85.57*

[a] Silage juice diluted 1:100.

Table 2. Percentage recovery of lactic acid calculated on standard lactic acid solutions added to two artichoke silage juices, using colorimetric method

Table 3. Percentage recovery of lactic acid calculated on standard lactic acid solutions added to artichoke silage juice, using HPLC method

Silage juice (*μg*)	*Solution added* (*μg*)	*Juice + solution added* (*μg*)	*Recovery* (%)
	0.0	607.3	–
	300.0	796.9	87.5
607.3	500.0	1008.6	90.8
	800.0	1403.0	99.5
	1000.0	1701.4	105.8

lactic acid,[5,10] due to their high accuracy and specificity. The relationship between the standard concentrations of lactic acid and the ratio lactic acid base peak area/internal standard area was significantly linear ($P<0.001$) ($y=-0.93+684.13x$, $R^2=0.9993$). In addition, this method showed high recovery percentages at ratios $>1000\,\mu g\,ml^{-1}$ (Table 3), high resolution (Fig 1), and the filtrates of silage samples could be analysed directly without being diluted. However, these methods require complex instrumentation and are not very suitable to handle a large number of samples at the same time.

Relationship between the methods

The relationship between the results of lactic acid content obtained by the colorimetric, HPLC and enzymatic methods are presented in a correlation matrix (Table 4). The enzymatic method was significantly correlated ($P<0.001$) to the colorimetric and HPLC assays ($R=0.9032$ and $R=0.9055$, respectively). However, the enzymatic method surpassed the results obtained by the colorimetric and HPLC methods.

The methods of measurement using high-performance liquid chromatography are used as a reference method,[16] as they are selective and accurate. The relationship between the results of the colorimetric and HPLC methods were significantly high ($P<0.01$) ($R=0.7169$), and the levels of lactic acid found by these two methods in silage juices were not different. The correlation between the HPLC and enzymatic methods was better than between HPLC and colorimetric methods. In the first case, the lactic acid contents were over-estimates for the four treatments but with the colorimetric method only the molasses treatment showed a much higher over-estimate.

Results from the present study suggest that the

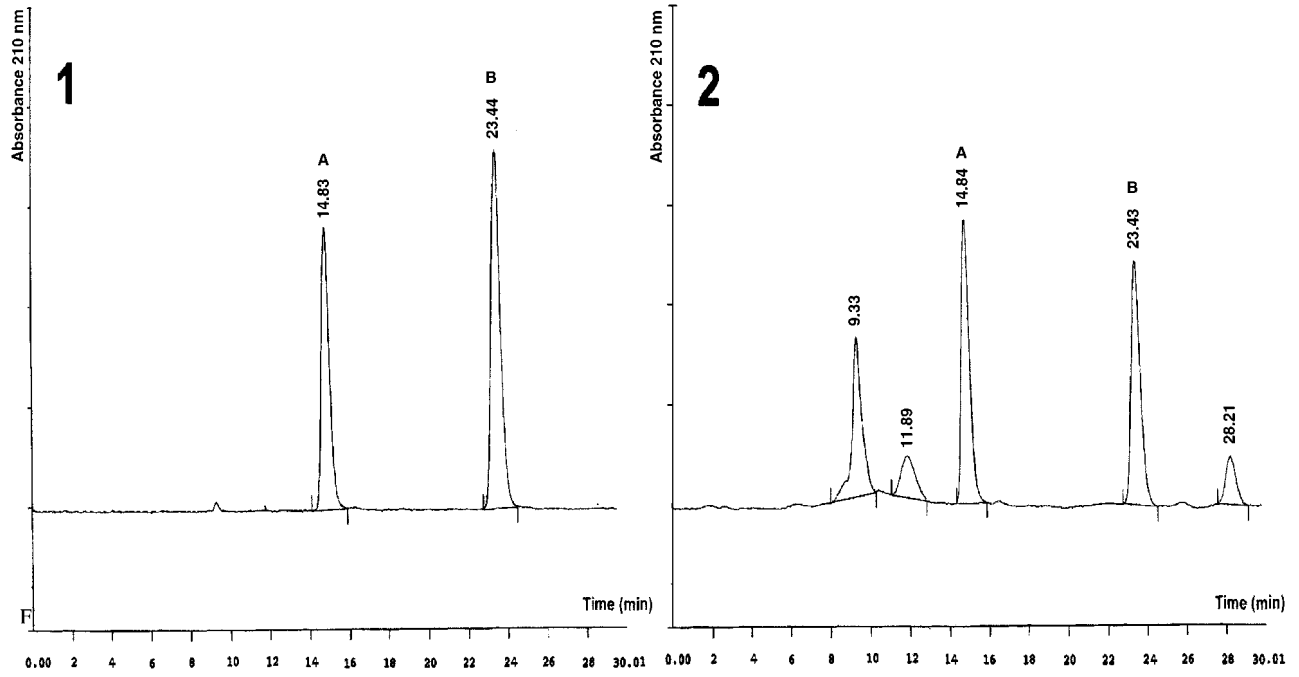

Figure 1. Chromatograms of lactic acid. (1) Standard solution of lactic acid with internal standard. (2) Filtrate of silage with internal standard. Peaks: A = citric acid (internal standard); B = lactic acid.

Table 4. Relationships (R) between the results of lactic acid content obtained by colorimetric, HPLC and enzymatic methods (correlation matrix)[a]

	Colorimetric method	*HPLC method*	*Enzymatic method*	*L(+) lactic acid*	*D(−) lactic acid*
Colorimetric method	1.0000	0.7965**	0.9032***	0.8941***	0.0941
HPLC method		1.0000	0.9055***	0.7169*	0.4622
Enzymatic method			1.0000	0.8894***	0.3068
L(+) lactic acid				1.0000	−0.1621
D(−) lactic acid					1.0000

[a] Significance: ***$P<0.001$; **$P<0.01$; *$P<0.05$.

colorimetric method can be used for the measurement of lactic acid in silages, to low concentration, with no important adverse presence of interfering compounds that would affect the results or the over-estimate values obtained by a reference method such as HPLC.

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