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Monitoring of the Fermentation Media of Citric Acid by the Trimethylsilyl Derivatives of the Organic Acids Formed

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In this approach, a derivatization method is described for monitoring of organic acids in fermentation media without any separation step. The aqueous phase of fermentation media was evaporated and heated in a silylation reagent to form trimethylsilyl (TMS) derivatives. The silylated compounds are analyzed by ^{29}Si nuclear magnetic resonance (^{29}Si NMR) and gas chromatography–mass spectrometry (GC-MS). ^{29}Si NMR can qualitatively monitor the components produced in the Krebs cycle. Quantification of these compounds is investigated by using selected ion monitoring mode of mass spectrometry. In this mode, mass to charge (m/z) values of their $[M - 15]^+$ ions, which are 465, 275, 247, 221, 335, 251, and 313 of TMS derivatives of citric, α-ketoglutaric, succinic, fumaric, L-malic, oxaloacetic, and palmitic (as an internal standard), acids, respectively, are used. The limit of detection and the linear working range for derivatized citric acid were found to be 0.1 mg L^{-1} and 10–3 × 10^4 mg L^{-1}. The relative standard deviation of the method for five replicates was 2.1%. The average recovery efficiency for citric acid added to culture media was ~97.2%. Quantitative results of GC-MS are compared with those obtained by an ultraviolet–visible method.

KEYWORDS: Citric acid; trimethylsilyl derivatives; gas chromatography–mass spectrometry; ^{29}Si NMR; *Aspergillus niger*

INTRODUCTION

Citric acid, a tricarboxylic acid intermediate in the Krebs cycle, is widely used in food and pharmaceutical industries. Because of its commercial and academic importance, the biosynthesis of citric acid by submerged fermentation using the filamentous fungus, *Aspergillus niger*, has been the subject of numerous investigations (*1–4*). On the other hand, the monitoring and controlling of the fermentation process, when a new species of *A. niger* was used, are very important. In fact, improvements in process control can be expected by complete identification of components by increasing the reproducibility and accuracy of measurements (*5*).

A large number of methods for the determination of citric and other organic acids from various sources have been reported. These include gravimetry analysis (*6*, *7*), potentiometry and biosensors (*8*, *9*), high-performance liquid chromatography (HPLC) (*10–14*), enzymatic flow injection (*15–18*), chemiluminescence methods (*19*, *20*), capillary electrophoresis (*21*, *22*), and spectrophotometric methods (*23*, *24*). Analytical derivatization reactions may find application in virtually any area of work involving analysis of organic compounds, particularly in work involving biological samples. Silyl derivatives are also formed by replacement of active hydrogen on OH, SH, and NH groups. Silylation of organic acids not only reduces the boiling points for GC analysis but also produces high molecular weight derivatives suitable for detection in mass spectrometry. Also, these derivatized compounds can be monitored by ^{29}Si nuclear magnetic resonance (^{29}Si NMR).

In a previous work, we determined citric acid in fermentation media by a pyrolysis–mass spectrometry (Pyr-MS) method (*25*). In the present work, ^{29}Si NMR and GC-MS techniques have been employed as simple and sensitive methods to monitor the citric acid and other Krebs cycle compounds produced in fermentation media. These methods require preparation of trimethylsilyl (TMS) derivatives of these compounds. ^{29}Si NMR and GC-MS peaks of TMS derivatives of citric, α-ketoglutaric, succinic, fumaric, L-malic, and oxaloacetic acids appear by their production and are eliminated by reducing in fermentation media. Also, TMS derivatives provide information on the chemical structure of compounds with a number of fragment ions, including $[M - 15]^+$ ions specified for each metabolite for quantitative works by GC-MS method.

MATERIALS AND METHODS

Instrumentation. All nuclear magnetic resonance measurements were carried out on a Bruker DRX 500 Avance spectrometer with field

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strength of 11.744 T. At this field, Si-29 resonates at 99.352 MHz. Tetramethylsilane (TMS) was used as an external reference. All chemical shift measurements were carried out at a probe temperature of 25.0 ± 0.1 °C; typical acquisition parameters were 400 ppm sweep width, 32 scans, 50 s relaxation delay, 0.83 s acquisition time, and 10 ms pulse width (90° pulse) with proton decoupling.

A Shimadzu GC-MS model QP-1100 EX was used. The gas chromatograph was a Shimadzu 14-A equipped with a fused silica capillary column CBP-5 (25 m × 0.22 mm i.d. with a film thickness of 0.25 μm) as the stationary phase. Helium was used as the carrier gas, at a constant flow rate of 1 mL min^{-1}. The temperature of the injection was 220 °C, and the oven was programmed from 150 to 220 °C at a rate of 10 °C min^{-1}; the final temperature was held for 5 min. TMS derivatives were analyzed in the EI mode using an ion source temperature at 250 °C and pressure of 5×10^{-6} Torr. Ionization was performed at 70 eV.

A Shimadzu UV-2100 spectrophotometer was employed for measurement of absorption of the citric acid chromophore at 428 nm. Two water baths were used, one thermostatically controlled at 32 °C and the other containing an ice−water slurry.

Reagents and Chemicals. Citric acid anhydride, α-ketoglutaric, succinic, fumaric, L-malic, oxaloacetic, and palmitic acids, hexamethyldisilasane (HMDS), trimethylchlorosilane (TMCS), and tetrahydrofuran (THF) were purchased from Merck (Darmstadt, Germany). Pyridine, acetic anhydride, potassium ferrocyanide, and trichloroacetic acid (TCA) were obtained from Fluka (Buchs, Switzerland). Beet molasses was obtained from Shazand Sugar Factory (Shazand, Iran). *Aspergillus niger* PTCC 5010 (Persian Type Culture Collection) was used throughout the experiment. The strain was maintained on potato dextrose agar (PDA) slants at 4 °C and subcultured at intervals from 2 to 3 months.

Standard Solutions. A stock solution of citric acid (50 mg mL^{-1}) was prepared by dissolving exactly 2.5000 g of citric acid anhydride in 50 mL of THF solvent. Also, a stock solution containing 2.5 mg mL^{-1} of palmitic acid was prepared by dissolving exactly 0.1250 g of palmitic acid in 50 mL of THF solvent. To prepare standard working solutions at six different concentrations in the range of 10−50000 mg L^{-1} with respect to citric acid, appropriate volumes of the citric acid stock solutions were accurately dispensed into 25-mL calibrated flasks. Then 1 mL of palmitic acid stock solution, as an internal standard, was added to each flask and finally diluted to the mark with THF as solvent. Thus, concentration ratios of 0.1, 1, 10, 100, 300, and 500 of citric acid to palmitic acid were obtained. For the UV−vis spectroscopy method, other standard working solutions at six different concentrations in the range of 1−300 mg L^{-1} with respect to citric acid were prepared with accurately dispensed appropriate volumes of the citric acid stock solution, into 50-mL calibrated flask. Then 20 mL of TCA was added to each flask and finally diluted to the mark with a 20% molasses solution.

Derivatization. To perform silylation, exactly 5 mL of above standard solutions dispensed into test tubes and appropriate amounts of HMDS and TMCS (as catalyst) were added to each tube. Then the resultant solutions were heated at 70 °C for 4 h and the tubes cooled to room temperature before measurement by ^{29}Si NMR and GC-MS.

Derivatization for Spectrophotometric Method. To perform this experiment, 1.0 mL of related standard working solutions was pipetted into labeled tubes that were then placed in an ice−water bath. Then 1.7 mL of pyridine was added to each tube, and subsequently 5.8 mL of acetic anhydride was immediately added to tubes. All tubes were placed in a 32 °C water bath for 30 min and then at room temperature for 20 min. The absorbance of the resultant chromophore was obtained at 428 nm. The calibration curve was constructed by plotting the absorbance against the concentration.

Fermentation Condition. Beet molasses were diluted with distilled water to give a concentration of 20% (w/v). A conical flask (500 mL) containing 100 mL of beet molasses, pH 7, was autoclaved at 121 °C and a pressure of 15 psi for 15 min. The medium was treated, while hot, with potassium ferrocyanide (10% w/v) to precipitate the heavy metals and then cooled to 30 °C. Spores of 5-day-old culture of *A. niger* grown on PDA were harvested and suspended in distilled water to obtain 1×10^{8} spores mL^{-1}. Five milliliters of spore suspension was transferred to the molasses solution (above flask), incubated at 30 °C, and agitated at 120 rpm until the fermentation process was completed.

Table 1. Characteristic ^{29}Si NMR Peaks of TMS Derivative Organic Acids Produced in the Krebs Cycle

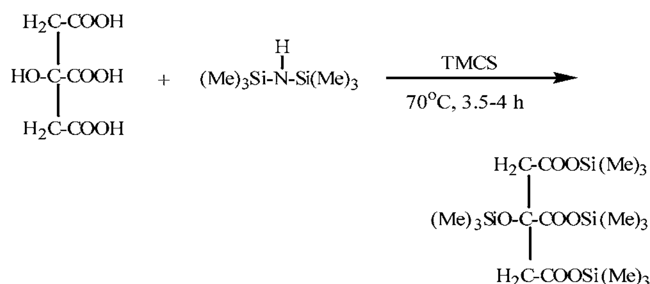
	acid					
	citric	fumaric	L-malic	succinic	oxalo-acetic	α-keto-glutaric
^{29}Si NMR position (ppm)	12.4	25.7	19.2	23.0	27.1	24.1

Preparation of Fermentation Samples for Analysis. In spectrophotometric methods, owing to its small linear working range (1−300 mg L^{-1}), dilution of samples is required. Thus, 1.0 mL of the aqueous phase of the fermentation medium was accurately dispensed into 50-mL calibrated flasks. Twenty milliliters of TCA (30% w/v) was added to each flask and then diluted to the mark with molasses solution (20% v/v), shaken, and left to stand for 30 min. The reason for adding TCA is to remove plant proteins present in sample and to obtain more reproducible results. Finally, 1 mL of this solution is analyzed by UV−vis spectroscopy.

To perform the analysis at different times from the fermentation process, exactly 5 mL of the aqueous phase of the fermentation medium was transferred to test tubes and evaporated. Because silylation reagent and TMS derivatives both are hydrolytically unstable, they must be protected from moisture. Then they were supplemented with an internal standard (for GC-MS), silylated, and analyzed as described above. For the ^{29}Si NMR analysis, 300 μL of the sample was transferred to an NMR tube and 200 μL of deuterated DMSO added.

RESULTS AND DISCUSSION

The medium of fermentation is a mixture of molasses, fungus, and citric and other organic acids in which they are produced and reduced based on the Krebs cycle during the fermentation process. Citric acid has four active hydrogens as part of its structure, and they are replaced with the trimethysilyl groups according to the reaction



The TMS-derivatized citric acid has a good volatility and thermal stability, making it amenable to GC-MS determinations as well as ^{29}Si NMR spectroscopy.

TMS derivatives of citric, α-ketoglutaric, succinic, fumaric, L-malic, and oxaloacetic acids have characteristic ^{29}Si peaks as shown in **Table 1**.

The characteristic ^{29}Si NMR peaks may be used for monitoring a fermentation medium during the process. **Figure 1** shows the ^{29}Si NMR spectra of TMS-derivatized compounds in the fermentation media at the 5th, 7th, and 10th days. On the 5th day of the fermentation processes, only the ^{29}Si NMR peaks of TMS-citric acid at 12.4, 23.4, and 25.8 ppm are seen. On the 7th day, the TMS-α-ketoglutaric acid peak appears at 24.1 ppm and the TMS-succinic acid peak at 23.0 ppm. On the 10th day the TMS-α-ketoglutaric and TMS-succinic acids peaks disappear

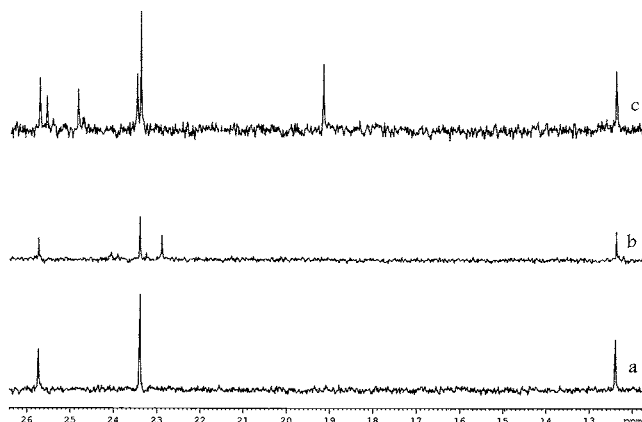


Figure 1. ^{29}Si NMR spectra of TMS derivative components on the (**a**) 5th, (**b**) 7th, and (**c**) 10th days of the fermentation processes.

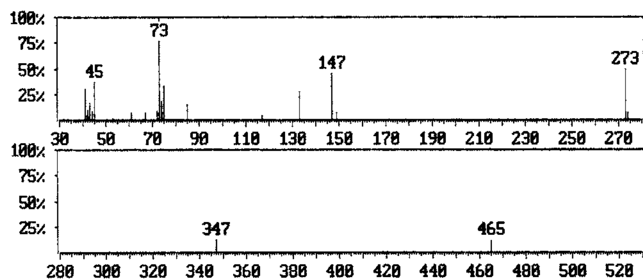


Figure 2. Full MS spectrum of the TMS derivative citric acid.

and TMS-L-malic and fumaric acids peaks appear at 19.2 and 25.7 ppm, respectively.

When GC analysis of the fermentation media is difficult or impossible due to the nonvolatile components in the fermentation media, the TMS derivatives of nonvolatile compounds can solve this problem. When TMS-citric acid is introduced to the ionization source, the fragmentation gives a pattern of peaks at *m*/*z* 147, 273, 347, and 465 as shown in **Figure 2**, which are used for qualitative and quantitative analysis.

As shown in the results, *m*/*z* 465 belongs to $[M - 15]^+$, which is a fragment ion of TMS-derivatized citric acid that has high abundance. This ion has good sensitivity and abundance, thus monitoring and quantification of citric acid can be performed using this fragment ion. The specificity of the method also increases because this ion appears at a high *m*/*z* value (the spectrum is very simple at *m*/*z* >100).

We noticed that selection of an internal standard (in the present work, TMS-derivatized palmitic acid) could recalibrate the system continuously and overcome any drift problems of the GC-MS data. Palmitic acid has an active carboxyl group and is easily converted to a trimethylsilyl derivative. **Figure 3** shows that when TMS-palmitic acid is introduced in the ionization source, it is subjected to fragmentation and an ion with *m*/*z* 313 is formed, which is assigned to the $[M - 15]^+$. The palmitic acid derivative is chosen as internal standard for several reasons. First, this compound after silylation has an *m*/*z* of 313, which does not overlap with any of the peaks of the compounds. Second, it does not react with the citric acid derivative and other compounds in the fermentation medium. It has high thermal stability and volatility and also shows a good abundance and proved to be reproducible in *m*/*z* 313.

According to the Krebs cycle, *A. niger* fungus may produce other organic acids such as fumaric, malic, succinic, α-keto-glutaric, and oxaloacetic acids as well as citric acid. These byproducts are able to react with the silylation reagent and produce the corresponding TMS-derivatized products. The *m*/*z* $[M - 15]^+$ of these organic acids could not interfere with each other at all and can be determined for quantitative works in the fermentation media.

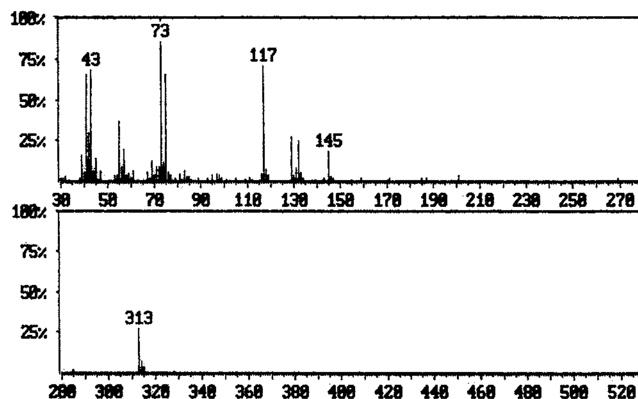


Figure 3. Full MS spectrum of the TMS derivative palmitic acid.

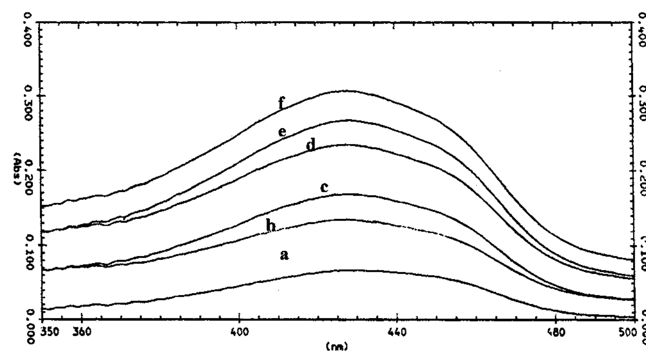


Figure 4. UV spectra of standard solutions of citric acid at different concentrations: (**a**) 50, (**b**) 100, (**c**) 150, (**d**) 200, (**e**) 250, and (**f**) 300 mg mL^{-1} of citric acid.

Table 2. Comparison of the GC-MS and UV Results

characteristic	GC-MS method	UV
linearity (mg L^{-1})	10–3 × 10^4	1–300
RSD[a]	2.1	1.94
LOD[b] (mg L^{-1})	0.1	13.2
performance time	~5 h	~90 min
R^{2} [c]	0.9967	0.9908
recovery (%)	97.2	95.2

[a] Relative standard deviation. [b] Limit of detection. [c] Correlation coefficient square.

As mentioned before, UV−vis spectroscopy is a routine method for the measurement of citric acid, suffering from drawbacks (*23*, *24*). The UV−vis spectra of citric acid are shown in **Figure 4**.

However, this method was used as a reference method to compare with the performance of our GC-MS approach. Results of this comparison are presented in **Table 2**.

The UV method is nonspecific and tedious for the analysis of citric acid in a complex matrix such as fermentation media. The requirement of large amounts of expensive, toxic solvents (pyridine and acetic anhydride), which can be harmful to the environment and have narrow linear working ranges, are some of the drawbacks of this method. The results in **Table 2** show that the LOD, LDR, RSD, and recovery efficiency of the GC-MS method are better than those of the UV method. Also, each of the acids formed in the fermentation media has a distinctive fragmentation pattern that allows easy identification. In addition, without utilizing any separation steps for organic acids from

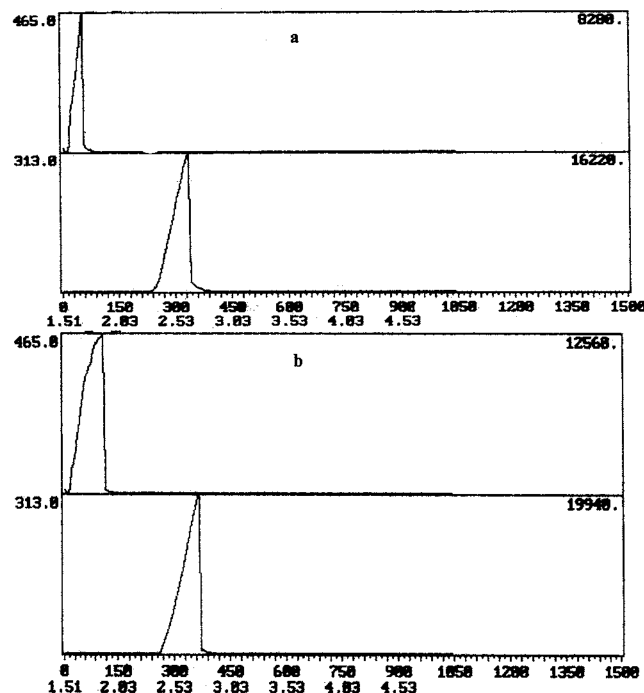


Figure 5. SIM chromatograms of the fermentation medium at the (**a**) 7th and (**b**) 8th days of the fermentation process.

Table 3. Recovery Test for the GC-MS Method

sample	citric acid added (g)	determined (g)	recovery %
1	0.100	0.098	98
2	0.050	0.0488	97.6
3	0.010	0.0096	96.1

fermentation media, the GC-MS method with SIM mode, using *m/z* 465 related to citric acid, allows a selective monitoring and quantifying. The equation of the calibration curve, of the fermentation medium, is $y = 0.6993x - 0.0079$, with $R^2 = 0.9967$. As an example, the SIM peak of citric acid and palmitic acid at two different fermentation times are shown in **Figure 5**.

On the basis of the calibration data used to compute the concentration of citric acid, the ratios of the peak areas representing the TMS-derivatized citric acid (*m/z* 465) to those of the internal standard (*m/z* 313) were calculated.

Precision and Recovery. The precision of the method was determined using standard solutions. After derivatization, samples were analyzed by five replications by GC-MS. Measurement of the response ratios of the analytes to the internal standard was repeatable with ~2.1% RSD.

To determine the method recovery for citric acid, known amounts of citric acid and known amounts of the internal standard (palmitic acid) were added to a 20% molasses solution. After derivatization, ~2 μL of the samples was injected in the GC-MS in five replicates and analyzed under the mentioned conditions. The results are shown in **Table 3**. The value of recovery is always >96%.

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