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Quantification of Organic Acids in Fermented Shrimp Waste by HPLC

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Summary

This work describes a simple, rapid, and reliable HPLC method for the determination of organic acids in fermented shrimp waste. Lactic, acetic and citric acids were quantified by HPLC with UV detection, on a 250×4.6 mm Extrasil ODS 5-μm column, mobile phase was ultrapure water adjusted with metaphosphoric acid to pH=2.1, flow rate 0.6 mL/min, column temperature 30 °C, and detection wavelength 210 nm. Under these conditions, the recovery (97.5 %) and the method repeatability (RSD=6.2 %) for lactic acid were of satisfying quality. Organic acids can preserve the quality and nutritive value of fermented shrimp waste.

Key words: organic acids, HPLC, lactic acid fermentation, shrimp waste

Introduction

Shrimp, mainly of genus *Penaeus*, is a major sea food in Sonora, Mexico. It is estimated that less than half of the animal resources ends up as products for human consumption. The remainder is classified as waste and much of it, in particular cephalothorax and exoskeleton, is sent to landfill sites, and represents an environmental problem (*1*). Shrimp waste has been the primary source of a natural biopolymer known as chitin (*2*). Additionally, it may be used as a source of carotenoid pigments (*3*) and protein with excellent amino acid profile (*4*). Sun drying is commonly applied to preserve this waste, but this is often carried out under unhygienic conditions leading to a product with high microbial loading (*5*). On the other hand, the ensiled formic acid can improve the nutritional quality of the resulting product (*6*). The use of proteolytic enzymes is a simple and inexpensive alternative method for the preservation and deproteinization of shrimp waste (*7*). However, prompt preservation through lactic acid bacterial fermentation, which has been used successfully in fish isolation, could be desirable as an alternative to sun drying, formic acid isolation or enzymatic methods (*8*). When shrimp waste is fermented by adding lactic acid bacteria and a carbohydrate source, acidification occurs, which lowers the pH and dissolves the calcium carbonate, while proteolysis carried out by the enzymes from the shrimp viscera and the fermented waste forms a silage containing a protein-rich liquor, a lipid fraction and the fairly clean insoluble chitin (*9*). The efficiency of lactic acid bacteria fermentation depends on factors such as the quantity of inoculum, glucose, initial pH and pH during fermentation, the amount and type of acid used, and fermentation time (*10,11*). Previous work in this area has been proven and optimized successfully (*12*), and the lactic acid production can be used to monitor the efficiency of fermentation and to measure the demineralization and deproteinization from shrimp shell (*13*).

Organic acids are found in food as a result of metabolism of large molecular mass compounds, such as carbohydrates, lipids, and proteins (*14*). These organic acids play an important role in the taste and aroma of dairy products and some authors use the level of organic acids to monitor starter activity and bacterial

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growth. Additionally, these acids can indicate the kind of fermentation (*15*). A commonly used method for the determination of acid content is alkaline titration using an appropriate visual indicator. However, the titration methods are generally not selective nor sufficiently sensitive or precise to detect a small acid content (*16*). To avoid these problems, gas chromatography and high-performance liquid chromatography (HPLC) methods have gained importance in organic acid analysis. Because of the speed, selectivity, sensitivity, reliability, and simple sample preparation methods involved in HPLC, these are the most widely used techniques. HPLC has been used with refractive index (*17*), UV detection (*18–25*), and pulsed electrochemical detection (*16*). Today, the reversed phase HPLC methods are very popular in food analysis; however, the available information on the organic acid content in samples of fermented shrimp waste is very limited (*26*).

Here, we report an HPLC-UV procedure to determine the mass fraction of organic acids in fermented shrimp waste, with the aim of monitoring the lactic acid fermentation during chitin production.

Materials and Methods

Reagents and chemicals

Standards of the lactic, acetic and citric acids were purchased from Supelco (Bellefonte, PA, USA). Metaphosphoric, citric, acetic and lactic acids, and sodium hydroxide were obtained from Productos Químicos Monterrey (Monterrey, N.L., Mexico). Ultrapure water was prepared using a NANOpure Diamond UV system (Barnstead International, Dubuque, IA, USA). All reagents used were of analytical grade.

Standards and quantification

Standard stock solutions of lactic acid (5.4 mg/mL), acetic acid (6.8 mg/mL) and citric acid (5.2 mg/mL) were prepared in ultrapure water and stored at 4 °C. The purity of all the reference standards was ≥99.9 %. Working solutions were prepared from these solutions and diluted with ultrapure water prior to analysis. For determination of lactic, acetic and citric acids in liquor fraction, the stock solution was analyzed together with the samples in all cases, and the quantification of the three analytes was performed building the calibration curves described in Table 1. All samples were analyzed in duplicate.

Table 1. Calibration parameters for the different organic acids analyzed

Organic acid	γ/(μg/mL)	Equation	R^2
Lactic	4.3–5400	y=1356.9x+1977.4	0.9992
Acetic	5.4–6840	y=1075.9x+102564.7	0.9994
Citric	4.1–5160	y=2081.9x+9748.2	0.9997

x=γ/(μg/mL); y=peak area; R^2=determination coefficient

Production of fermented shrimp waste

Shrimp (*Penaeus* spp.) waste (heads and cephalothorax) samples were collected from local shrimp processing factories in South Sonora, Mexico. The waste was packed in plastic bags and stored at –20 °C before analysis. Slightly thawed minced waste (500 g) was placed into 1000-mL glass flasks and mixed with 10 % (by mass) cane sugar and 5 % (by volume per mass) commercial inoculum (absorbance A=1.7, measured at 535 nm), stirred, and incubated in a water bath at 30 °C for 36 h. At the start of the fermentation the pH was adjusted to 6.0 with 2 M citric acid or 5 M acetic acid or 5 M lactic acid. The pH of the extract obtained during fermentation was measured in 10 mL of the sample using a potentiometer (Corning 340, NY, USA). In preliminary assays, the fermentation was conducted with and without constant stirring to examine its possible effects on chitin extraction. Based on that, the fermentation was carried out with continuous stirring. The silage was centrifuged at 6440×g for 15 min at 5 °C to obtain a chitin-rich fraction (sediment), the protein-rich liquor, and the lipid fraction. The protein-rich liquor (aqueous phase) was separated, and stored at 4 °C.

HPLC equipment

The equipment used in this research was an HPLC-UV system (GBC Scientific Equipment, Dandenong, Australia) equipped with an auto injector LC1650, an on-line solvent degasser LC1460, a system controller WinChrom, a pump LC1150, a column oven LC1150, a 20-μL injection loop (Rheodyne, Cotati, CA, USA), and a photodiode array detector LC5100.

Chromatographic conditions

Chromatographic analysis was performed using an analytical SS Exil ODS column (25×0.4 cm i.d.) with a particle size of 5 μm (SGE Analytical Science, Dandenong, Australia). The analyses were carried out isocratically at a flow rate of 0.6 mL/min, employing as mobile phase water adjusted to pH=2.1 with metaphosphoric acid. The column was thermostated at 30 °C. Injection volume was 20 μL. Organic acids were detected at 210 nm. Lactic, acetic and citric acids were identified by retention and spectral data. The total time between injections was 45 min.

Sample preparation

Fermented samples were collected from laboratory batches of 500 g. Centrifugation was used to separate the aqueous phase (protein-rich liquor) from the sediment (chitin-rich fraction) and the lipid fraction. The upper phase (solid lipid fraction) was separated manually and aqueous phase was drained off, after that 2 mL of aqueous extract were placed in a volumetric flask and diluted to 25 mL with ultrapure water and sonificated for 2 min. The clear extract obtained was kept at –20 °C until analysis. This solution was filtered through a 0.45-μm cellulose acetate membrane, and finally, it was injected directly into the chromatograph.

We determined the water content of the aqueous extract obtained during the fermentation from shrimp waste by weighing the fraction before and after drying the fraction to a constant mass in an oven at 100 °C.

Statistical analysis

For statistical analyses, the computer program used was SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). The relative standard deviation (RSD) is the ratio of standard deviation to average value expressed as percentage.

Results and Discussion

The current methodology has been developed to overcome deficiencies in other published methods, particularly when measuring lactic acid. As noted in the Introduction section, traditionally, lactic acid is measured by two methods: titration and HPLC. Because of the simplicity of the method, titration is most commonly used, but it is inaccurate. On the other hand, in order to know the exact mass fraction of a specific acid, the HPLC method has been selected. In our laboratory, it is necessary to know the fermentation pattern of the shrimp waste; due to this we have studied a versatile and rapid HPLC methodology to analyze organic acids, mainly lactic acid from shrimp waste during fermentation.

Presence of organic acids in the fermented shrimp waste

There have been several studies published about lactic fermentation of shrimp waste, regarding the conditions for the extraction of chitin, astaxanthin and protein, but the differences between the diverse methods are rather insignificant (*3*,*10*,*13*). As noted in the Materials and Methods section, before fermentation, the pH of the samples was adjusted initially to 6.0 with different acids (acetic, citric or lactic) to avoid the growth of spoilage microorganisms and obtain a fermented shrimp waste of good quality. In addition, in this work homofermentative lactic acid bacteria were used as a commercial inoculum to ensure the rapid acidification of shrimp waste (*12*). The pH of the fermented shrimp waste silage dropped from 7.23 to 4.33 in the first 28 h. The rapid and sharp drop in the pH values indicated good fermentation of the shrimp waste. This pH value is within the recommended values for successful fermentation (pH=4.4 to 4.8) (*5*,*11*,*13*,*27*).

In preliminary experiments, acidified samples were analyzed at different times during fermentation, and all the samples had lactic acid as the main acid. In addition, the samples showed acetic or citric acids as minor components used for adjusting the pH. Acids in the samples were identified by comparison of retention times and the UV absorption spectra with those obtained from the corresponding standards. For determination of retention times, the reference standards were injected both individually and as a mixture. An example of an HPLC separation of the organic acids from fermented shrimp waste is shown in Fig. 1a (citric acid was not detected in this sample), while Fig. 1b shows the HPLC separation

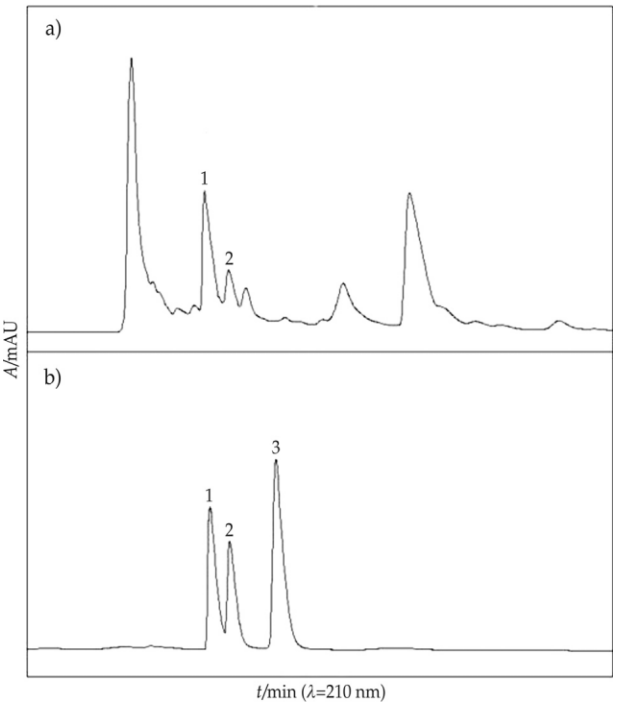

Fig. 1. HPLC chromatograms of: a) a mixture of standard solutions of organic acids, b) sample of an extract from fermented shrimp waste acidified with acetic acid. Peaks identified: (1) lactic acid, (2) acetic acid, and (3) citric acid

of standard solution. Peaks were observed at (8.057±0.06) min for lactic acid, (8.81±0.06) min for acetic acid, and (11.42±0.13) min for citric acid, for an average of 10 injections.

Sample preparation

There have been many studies published about conditions for extracting organic acids from different fermented food (*15*,*20*,*21*,*24*). For fermented shrimp waste, very little information is available in this area (*12*). To determine optimal sample amounts, preliminary assays were performed with 0.5, 1.0 and 2.0 mL of extract per 25 mL of ultrapure water with different time lengths of sonification (0, 1, 2 and 4 min). The process indicated that acceptable results are achievable with 2 mL of extract in 25 mL of ultrapure water and 2 min of sonification.

Method validation

It was observed from the preliminary results that lactic acid was the main organic acid produced during the fermentation, and we have chosen it to study the reproducibility and accuracy of the method.

Table 1 shows the linearity of standard curves expressed in terms of the determination coefficient (R^2) from plots of the integrated peak area *vs*. concentration of the standard (μg/mL). These equations were obtained from standard solutions of four different concentrations, selected as representatives of the range of concentrations in the aqueous extract from shrimp waste hydrolysate. Calibration curves were linear for all the organic acids investigated. Method reproducibility (between-run pre-

cision) for lactic acid was good (extraction and analysis of a single aqueous extract from fermented shrimp waste sample was tested 8 times, in each case with 2 sample injections, relative standard deviation RSD=6.2 %, and the average value was 246.8 mg lactic acid/g dry mass), which is sufficient for routine analysis of this acid in quality control laboratories. Accuracy was estimated by means of recovery assays. The recovery of the method was evaluated by the analysis of 8 samples from aqueous extract, which were spiked with a known mass fraction of lactic acid (377.1 mg/g dry mass) before extraction and quantitation. In the spiked samples, the mass fraction of lactic acid was approx. 614.7 mg/g dry mass, and the obtained value of 97.5 % (RSD=2.4 %) was good, considering the concentration and type of analyte. Determination of the detection limit for lactic acid (three times the basis of signal-to-noise ratio) was 1 μg/mL. It is not possible to compare these results with other works, because this is the first time that a study has been carried out on aqueous extract from fermented shrimp waste.

Mass fractions of lactic, acetic and citric acids in aqueous extract from fermented shrimp waste

The practical applicability of the method was assessed by the analysis of 18 samples from aqueous extract obtained from different batches of fermented shrimp waste during monitoring of fermentation (Table 2). There was a wide range of organic acid mass fractions; for example, lactic acid ranged from a few mg/g to about 200 and 300 mg/g of dry mass in the last part of fermentation. This common pattern was present in all samples; lactic acid mass fraction increased after 8 h of fermentation, but the other acids showed higher mass fractions for only 4 h and then declined through the rest of the fermentation. Likewise, acetic and citric acids were only detected in the samples acidified with these acids. The acetic acid mass fraction ranged from 5.0 to 57.3 mg/g dry mass, and the citric acid mass fraction ranged from 5.4 to 92.7 mg/g of dry mass. In relation to this, lactic acid has been reported in the range from 85 to 177 mg/g dry mass for grass silages (*28*), 60.0 to 71.2 g/100 g for delignified cellulosic material (*29*), and 62.7 mg/g wet basis for shrimp waste silage (*12*).

Table 2. Lactic, acetic and citric acid mass fractions in fermented shrimp waste

Batch acidified with	w/(μg/g dry mass)		
	Lactic acid	Acetic acid	Citric acid
Acetic acid	12.2	17.5	N.D.
	15.9	57.3	N.D.
	210.5	5.0	N.D.
	12.2	24.2	N.D.
	39.4	13.2	N.D.
	78.1	22.9	N.D.
Lactic acid	130.6	N.D.	N.D.
	163.2	N.D.	N.D.
	302.6	N.D.	N.D.
	154.2	N.D.	N.D.
	234.6	N.D.	N.D.
	338.0	N.D.	N.D.
Citric acid	125.0	N.D.	92.7
	159.4	N.D.	16.5
	255.6	N.D.	5.4
	9.1	N.D.	75.9
	5.4	N.D.	4.5
	264.5	N.D.	23.4

N.D.=not detected

Conclusions

This work enables the implementation of an easy and reliable methodology for lactic, acetic and citric acid analysis in extracts of fermented shrimp waste. In addition, the proposed procedure can be used in routine analysis of fermented foods. Our results reveal a variation in lactic acid mass fraction during the processing of this waste. Organic acids can preserve the quality and nutritive value of fermented shrimp waste.

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References

1. M. Healy, A. Green, A. Healy, Bioprocessing of marine crustacean shell waste, *Acta Biotechnol. 23* (2003) 151–160.
2. A. Percot, C. Viton, A. Domard, Characterization of shrimp shell deproteinization, *Biomacromolecules, 4* (2003) 1380–1385.
3. R. Armenta-López, I.L. Guerrero, S. Huerta, Astaxanthin extraction from shrimp waste by lactic fermentation and enzymatic hydrolysis of the carotenoprotein complex, *J. Food Sci. 67* (2002) 1002–1006.
4. J. López-Cervantes, D.I. Sánchez-Machado, J.A. Rosas--Rodríguez, Analysis of free amino acids in fermented shrimp waste by high-performance liquid chromatography, *J. Chromatogr. A, 1105* (2006) 106–110.
5. O.A. Fagbenro, Preparation, properties and preservation of lactic acid fermented shrimp heads, *Food Res. Int. 29* (1996) 595–599.
6. L.C. Nwanna, Nutritional value and digestibility of fermented shrimp head waste meal by African catfish *Clarias gariepinus*, *Pak. J. Nutr. 2* (2003) 339–345.
7. N. Gagné, B.K. Simpson, Use of proteolytic enzymes to facilitate the recovery of chitin from shrimp wastes, *Food Biotechnol. 7* (1993) 253–263.
8. G.M. Hall, S. De Silva: Lactic Acid Fermentation of Scampi (*Penaeus monodon*) Waste for Chitin Recovery. In: *Advances in Chitin and Chitosan*, C.J. Brine, P.A. Sandford, J.P. Zikakis (Eds.), Elsevier Applied Science, London, UK (1992) pp. 633–668.
9. G. Legarreta, Z. Zakaria, G.M. Hall: Lactic Fermentation of Prawn Waste: Comparison of Commercial and Isolated Started Cultures. In: *Advances in Chitin Science*, *Vol. 1*, A. Domard, C. Jeuniaux, R. Muzzarelli, G. Roberts (Eds.), Jacques André, Lyon, France (1996) pp. 399–406.

10. M.S. Rao, J. Muñoz, W.F. Stevens, Critical factors in chitin production by fermentation of shrimp biowaste, *Appl. Microbiol. Biotechnol. 54* (2000) 808–813.

11. M.S. Rao, W.F. Stevens, Fermentation of shrimp biowaste under different salt concentrations with amylolytic and non-amylolytic *Lactobacillus* strains for chitin production, *Food Technol. Biotechnol. 44* (2006) 83–87.

12. K. Shirai, I. Guerrero, S. Huerta, G. Saucedo, A. Castillo, R.O. González, G.M. Hall, Effect of initial glucose concentration and inoculation level of lactic acid bacteria in shrimp waste ensilation, *Enzyme Microb. Technol. 28* (2001) 446–452.

13. L.A. Cira, S. Huerta, K. Shirai, Lactic fermentation of shrimp heads (*Penaeus* sp.) in a solid-state fermentation reactor, *RMIQ, 1* (2002) 45–48.

14. H.G. Daood, P.A. Biacs, M.A. Dakar, F. Hajdu, Ion-pair chromatography and photodiode-array detection of vitamin C and organic acids, *J. Chromatogr. Sci. 32* (1994) 481–487.

15. P. Manolaki, M.C. Katsiari, E. Alichanidis, Effect of a commercial adjunct culture on organic acid contents of low-fat Feta-type cheese, *Food Chem. 98* (2006) 658–663.

16. I.G. Casella, M. Gatta, Determination of aliphatic organic acids by high-performance liquid chromatography with pulsed electrochemical detection, *J. Agric. Food Chem. 50* (2002) 23–28.

17. J.K. Palmer, D.M. List, Determination of organic acids in foods by liquid chromatography, *J. Agric. Food Chem. 21* (1973) 903–906.

18. M.O. Nisperos-Carriedo, B.S. Buslig, P.E. Shaw, Simultaneous detection of dehydroascorbic, ascorbic and some organic acids in fruits and vegetables by HPLC, *J. Agric. Food Chem. 40* (1992) 1127–1130.

19. M.L. Vázquez-Oderiz, M.E. Vázquez-Blanco, J. López-Hernández, J. Simal-Lozano, M.A. Romero-Rodríguez, Simultaneous determination of organic acids and vitamin C in green beans by liquid chomatography, *J. AOAC Int. 77* (1994) 1056–1059.

20. G. Zeppa, L. Conterno, V. Gerbi, Determination of organic acids, sugars, diacetyl, and acetoin in cheese by high-performance liquid chromatography, *J. Agric. Food Chem. 49* (2001) 2722–2726.

21. M. Kordiš-Krapež, V. Abram, M. Kač, S. Ferjančič, Determination of organic acids in white wines by RP-HPLC, *Food Technol. Biotechnol. 39* (2001) 93–99.

22. S. Suárez-Luque, I. Mato, J.F. Huidabro, J. Simal-Lozano, Solid-phase extraction procedure to remove organic acids from honey, *J. Chromatogr. B, 770* (2002) 77–82.

23. S.F. Chen, R.A. Mowery, V.A. Castleberry, G.P. van Walsum, C.K. Chambliss, High-performance liquid chromatography method for simultaneous determination of aliphatic acid, aromatic acid and neutral degradation products in biomass pretreatment hydrolysates, *J. Chromatogr. A, 1104* (2006) 54–61.

24. M. Erbaş, M.K. Uslu, M.O. Erbaş, M. Certel, Effects of fermentation and storage on the organic and fatty acid contents of tarhana, a Turkish fermented cereal food, *J. Food Compos. Anal. 19* (2006) 294–301.

25. B.M. Silva, P.B. Andrade, R.C. Martins, R.M. Seabra, M.A. Ferreira, Principal component analysis as tool of characterization of quince (*Cydonia oblonga* Miller) jam, *Food Chem. 94* (2006) 504–512.

26. L.A. Cira, S. Huerta, G.M. Hall, K. Shirai, Pilot scale lactic acid fermentation of shrimp wastes for chitin recovery, *Process Biochem. 37* (2002) 1359–1366.

27. W.J. Jung, J.H. Kuk, K.Y. Kim, R.D. Park, Demineralization of red crab shell waste by lactic acid fermentation, *Appl. Microbiol. Biotechnol. 67* (2005) 851–854.

28. H. Danner, L. Madzingaidzo, M. Holzer, L. Mayrhuber, R. Braun, Extraction and purification of lactic acid from silages, *Bioresour. Technol. 75* (2000) 181–187.

29. O. Elezi, Y. Kourkoutas, A.A. Koutinas, M. Kanellaki, E. Bezirtzoglou, Y.A. Barnett, P. Nigam, Food additive lactic acid production by immobilized cells of *Lactobacillus brevis* on delignified cellulosic material, *J. Agric. Food Chem. 51* (2003) 5285–5289.