Iran Agricultural Research 17:125-138 (1998)

HPLC ANALYSIS OF ORGANIC ACIDS IN RECOMBINED IRANIAN FERMENTED WHITE CHEESE

SH. DOKHANI[1]

Department of Food Science and Technology, College of Agriculture, Isfahan University of Technology, Isfahan, Iran.

(Received: May 23, 1998)

ABSTRACT

Recombined milk products such as cheese are of importance in countries with low raw milk production or with difficulty of milk collection. Therefore, composition and physicochemical properties of such products would be of considerable value. In this study four types of Iranian recombined fermented white cheese were processed with skim milk powder plus butter oil with or without stabilizer, and ripened for two months at 13 ± 1°C in 10% salt solution. Organic acids of the cheese were determined by an HPLC equipped with SCR-101 H column and U.V. monitor at 210 nm using standards with more than 99.5% purity and percent recovery. Organic acids such as oxalic, orotic, citric, oxalacetic, succinic, lactic, formic, acetic, D-pyroglutamic, propionic, butyric, and hippuric were quantitatively determined in all samples. Results revealed different amounts compared with the results obtained for four types of natural cheeses processed in the pilot plant or prepared from the market. More acids were quantified in natural cheese samples than in recombined cheese. Formic acid was significantly higher in recombined than natural cheese. Hippuric acid was present only in one type of recombined cheese. Several other acids such as isocitric, cis-aconitic and alpha-ketoglutaric were only detected qualitatively in cheeses.

1. Associate Professor.

تحقیقات کشاورزی ایران

۱۷:۱۲۵-۱۳۸ (۱۳۷۷)

تجزیه کیفی و کمی اسید های آلی در پنیر های تخمیری سفید ایرانی و تهیه شده از شیر طبیعی ویا باز سازی شده

شهرام دخانی

دانشیار علوم و صنایع غذایی، دانشکده کشاورزی دانشگاه صنعتی اصفهان، اصفهان، ایران.

چکیده

فرآورده های شیری باز سازی شده از جمله پنیر درکشورهائی که با کمبود تولید شیر ویا مسائل مربوط با حمل و نقل مواجه هستند دارای اهمیت می باشند. بنابراین، اطلاع از ترکیب و خصوصیات فیزیکی و شیمیائی این گونه پنیر ها دارای ارزش زیادی است. در این تحقیق، چهار نوع پنیر تخمیری ایرانی، از شیر باز سازی شده با شیر خشک بدون چربی و روغن کره، همراه با تثبیت کننده یا بدون آن، تهیه گردید و به مدت ۲ ماه در آب نمک ۱۰ درصد در دمای ۱+۱۳ درجه سانتی گراد پرورانده شد. اسید های آلی پنیرهای باز سازی شده، با چهار نوع پنیر طبیعی ساخته شده در پایلوت پلنت ویا خریداری شده از بازار مقایسه شدند. تعیین اسید های آلی تولید شده در نمونه ها، با روش کروماتوگرافی مایع پیشرفته با استفاده از ستون مخصوص تعیین اسید های آلی SCR-101 H و در طول موج ۲۱۰ نانومتر با شناساگرفرا بنفش و در مقایسه با استاندارد های با خلوص و بازیابی ۹۹/۵ درصد انجام شد. اسید های آلی اگزالیک، اروتیک، سیتریک، اگزالواستیک، سوکسینیک، لاکتیک، فرمیک، استیک، دی- پیروگلوتامیک، پروپیونیک، بوتیریک و هیپوریک در نمونه ها اندازه گیری شد. اگر چه اسید لاکتیک به عنوان اسید غالب در نمونه ها اندازه گیری شد، اما اسید های آلی دیگر نظیر سیتریک، فرمیک، پروپیونیک و بوتیریک هم از نظر مقدار حائز اهمیت بودند. در مجموع ، میزان اسید های آلی

پنیر های طبیعی به مراتب بیشتر از پنیر های باز سازی شده بود. اسید آلی فرمیک در پنیر های باز سازی شده بطور معنی داری بیش از پنیر های طبیعی بود و اسید هیپوریک فقط در یک نمونه پنیر باز سازی شده یافت شد. چندین اسید آلی دیگر مانند ایزوسیتریک، سیس آکونیتیک و آلفاکتوگلوتاریک فقط از نظر کیفی در نمونه های پنیرتشخیص داده شد.

INTRODUCTION

The history and technology of recombined milk and related products such as cheese goes back to 1920's. After World War II and in 1950's the manufacturing processes and importance of recombined milk and cheese were much understood in the world, particularly, for African, Asian and other developing countries with low fresh milk production .

Recombined cheese is usually manufactured with a suitable recombined milk, low heat non-fat dried milk powder plus water and anhydrous milk fat. The milk formula is prepared at 40°C, homogenized, pasteurized and cooled to 32°C (3). The milk formula could also be prepared by correcting the salt and protein balances of a high-heat milk powder (4, 5, 8, 11, 16).

Organic acids in different foods are of value in determining the technological and nutritional quality, or the biochemical processes. Such compounds could also be very important in the shelf life of foods (1).

The biochemical processes in fermented milk and cheese or other food products produced from enzymes and microbial activities would provide a series of major or minor organic acids such as lactic, formic, acetic, propionic, butyric, etc. that at appropriate proportions affect the organoleptic quality.

Different methods of organic acid analysis such as volumetric, gravimetric, spectrophotometric, enzymatic, or chromatographic have been pursued. The high performance liquid chromatography (HPLC) has been the most successful method for qualification and quantification of organic acids in foods (1, 2, 7, 9, 10, 12, 13, 17).

The purpose of this research was to determine the different types of organic acids both qualitatively and quantitatively in different kinds of recombined cheese, manufactured from different formulated and recombined milks, and to compare the results with those for the natural fermented white cheese processed at the pilot plant of Food Science Department, Isfahan University of Technology, or the natural cheese purchased from the market.

MATERIALS AND METHODS

Materials

1. Non-fat dried milk (NFDM) and anhydrous milk fat was purchased from the Milk Pasteurization Factory of Isfahan, Iran.
2. Milk clotting enzymes, rennet and hannilase, or cheese starters, lactic acid bacteria, were received in vacuum dried packages (Dri-Vac.) from the CHR. Hansen Labs. of Copenhagen, Denmark.
3. All chemicals and standards (>99.5% purity) were ordered from Merck or Zigma Chemical Companies of Germany and England, respectively.
4. Fresh milk was obtained from Lavark Experimental Station of the College of Agriculture, Isfahan University of Technology.

Methods

HPLC system. A liquid chromatography Shimadzu LC-6A, equipped with a UV detector, Shimadzu SPD-6AV, a column oven model CTO-6A, a system controller model SCL-6A, and computerized analyzing Chroinatopack model C-R4A. and a Shimadzu ion exclusion column model SCR-101 H (7.9 mm i.d. and 30 cm l.) were used at an oven temperature of 75°C (15). UV detector was set at 210 nm. Organic acids were eluted from the column at flow rate of 0.7 ml min^{-1}. The chart speed was maintained at 6 mm per min. A mobile phase of HPLC grade sulfuric acid, 0.009 N, was run through the column.

Cheese processing. Recombined milk formulation: four types of recombined milk were formulated as follows: 1) 44 kg water + 4.5 kg non-fat dry milk (NFDM) + 1.5 kg anhydrous milk fat (AMF)+ 0.026 kg calcium hydrogen phosphate + 0.025 kg calcium chloride; 2) 44 kg water+4.5 kg NFDM + 1.5 kg AMF+0.025 kg calcium chloride; 3) 44 kg water+4.5 kg NFDM + 1.5 kg AMF+0.750 kg sodium caseinate+0.052 kg calcium hydrogen phosphate + 0.009 kg phosphoric acid; and 4) 44 kg water + 4.5 kg NFDM + 5 kg AMF as control (3).

The above types of recombined milk were mixed at 40°C and homogenized at two stages, with pressures of 70 and 35 kg cm^{-2} (1000 and 500 psi) at 65°C. The remaining process up to the cheese ripening was the same as for the natural fresh milk (3). Exactly 1% of lactic starter mixture of *Streptococcus, lactis, S. diacetylactis, S. thermophillus,* and *Lactobacillus bulgaricus* was used in each milk type. Milk clotting enzyme, hannilase, was used at a concentration of 1.4 g 100 kg^{-1} of milk. After ripening of brined white cheese (10% brine) for two months at 14°C, the 17 kg cans were opened and cheese cubes were wrapped in polyethylene bags and stored at -18°C for HPLC analysis. Samples were analyzed for moisture content at the time of organic acid analysis (3).

Extraction and organic acid analysis. The extraction procedure of Houlberg (6) was used with some modifications. One gram of well mixed cheese sample from one kg cheese cube was well homogenized with mortar and pestle in five ml buffer, pH 2.1, (500 ml 0.009 N sulfuric acid + 50 ml double distilled water+5 ml 5% EDTA). The homogenate was centrifuged at 3000 rpm for 30 min in an EBA-3 Hettrich centrifuge. The clear supernatant was filtered through 0.45 µm millipore filters and 1.0 to 10.0 µl of the filtrate was injected into HPLC using a Rheodyne injection port equipped with a 10 µl injection loop. The retention time and area of each peak resolved and detected were compared with those for organic acid standards for qualitative and quantitative determinations.

Standard Curves and Recovery Experiments

Twelve standards of organic acids (> 99.5% purity) were dissolved separately in double distilled water at four different concentrations . The

lowest concentration at the time of injection was for orotic acid , 0.0265 $\mu g\ \mu l^{-1}$ and the highest concentration was for lactic acid, 160.0 $\mu g\ \mu l^{-1}$. A standard mixture of 12 organic acids was prepared from stock solutions at different concentrations and one µl of fresh mixture was injected into the HPLC.

The recovery of organic acids from cheese sample was determined as follows: One gram of cheese sample was spiked with a known amount of a single standard organic acid, at the rate of 100 to 61000 $\mu g\ g^{-1}$ cheese. The samples were extracted and prepared as above. Recoveries were based on the difference between the total concentration of organic acid in the spiked versus the unspiked cheese samples.

RESULTS AND DISCUSSION

Recombined Cheese Processing

As in previous studies (3, 4, 8), addition of calcium salts to the recombined milk formula resulted in better coagulation during cheese making. Also, homogenization of the recombined milk caused no separation of butter fat from the milk formulas and, therefore, a better consistency and acceptability of the final cheese product was achieved. These findings support those by other investigators (11, Lablee and Malek, personal communication).

HPLC Analysis of Organic Acids in Cheese

A typical HPLC chromatogram of organic acids partitioned from recombined cheese sample No.3, is presented in Fig. 1.

The analysis of organic acids with HPLC revealed the presence of isocitric, oxalic, orotic, citric, oxalacetic, succinic, lactic, formic, acetic, D-pyroglutamic, propionic, and butyric acids in all cheese samples. Hippuric acid was present in only recombined cheese No. 3 (Figs. 1 and 2).

Other acids such as cis-aconitic and alpha-ketoglutaric (Fig. 2) were detected qualitatively in the samples. A few peaks remained unknown during the whole study. The retention times of standard organic acids and

cheese samples are shown in Table 1. All organic acids were partitioned by HPLC in 30 min. according to the ion exclusion chromatography, ionization coefficient values (pKa), and the electrostatic forces or polarity among stationary phase, mobile phase and organic acids. Wang *et al.* (17) and Marsilli *et al.* (9) reported several organic acids in cheese or other fermented dairy products that confirms the results of this study.

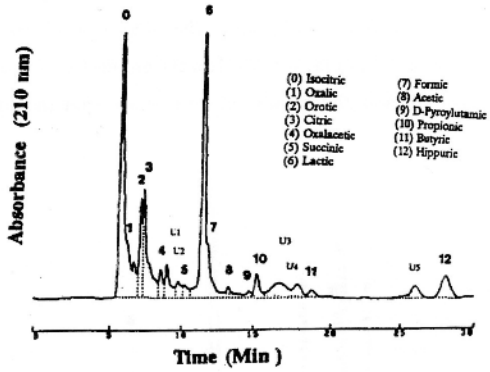

Fig. 1 A typical HPLC chromatogram of organic acids, partitioned from recombined cheese sample No. 3. Unknowns: retention times (min): U1= 8.90, U2 = 9.50, U3 = 16.58, U4 = 17.50 and U5 = 26.0.

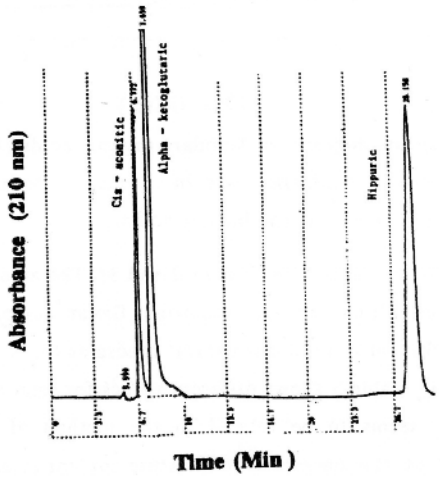

Fig. 2. HPLC chromatogram of standard organic acids (>99.5%): cis-aconitic, alpha-ketoglutaric, and hippuric acids.

The typical HPLC chromatogram of organic acid standard mixture used in this study is presented in Fig. 3. The retention time for the majority of acids (Table 1) was between 5.6 and 19.0 min. The quantities of different organic acids in recombined cheese samples are shown in Table 2. These values are comparable with the amounts determined in several types of natural cheeses processed in the pilot plant or prepared from the market (Table 3). Lactic acid was the major acid in all samples. Recombined cheese No.1 contained the lowest amount of lactic acid. Citric acid content of recombined cheese was higher than that in natural cheese

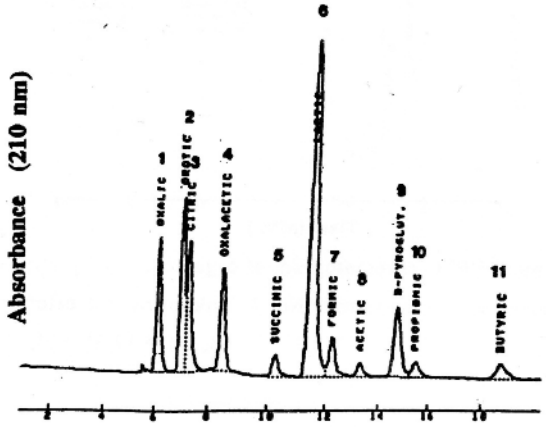

Fig. 3. HPLC chromatogram of standard organic acids (>99.5%): oxalic, orotic, citric, oxalactic, succinic, lactic, formic, acetic, D-pyroglutamic, propionic and butyric acids.

prepared in pilot or rural area (Tables 2 and 3). The amount of propionic and butyric acids which are very important flavor compounds in brined white cheese was also high in natural cheese as for recombined cheese (Tables 2 and 3). Table 3 shows different values for total acidity of cheese samples which seems to be related to the method of processing and ripening period of the cheese. The moisture content of cheese samples is shown in Table 4. Except industrial and recombined No.3 which contained 52.9 and 54.4 % moisture, respectively, the other samples had higher

moisture values. The Danish feta cheese with increased amount of lactic acid showed the highest total acidity of about 3%. Among the recombined cheeses, No.4 or control showed the highest amount of total acidity (Table 2). Orotic acid was present at almost the same quantity in all recombined and natural cheese samples. This organic acid has been reported (14) to be a hypocholesteremic agent in fermented dairy products.

Table 1. The retention times of organic acid mixtures analyzed from standards or cheese samples by HPLC.

Organic acids	Retention times (min)†	
	Standards	Range in cheese samples
Isocitric	5.50	5.56-5.65
Oxalic	6.09	6.08-6.14
Cis-Aconitic	6.77	6.65-6.69
Orotic	7.02	6.98-7.04
Citric	7.26	7.21-7.30
Alpha-Ketoglutaric	7.65	7.61-7.68
Oxalacetic	8.45	8.45-8.48
Succinic	10.25	10.21-10.28
Lactic	11.44	11.41-11.47
Formic	12.23	12.20-12.27
Acetic	13.28	13.24-13.30
D-Pyroglutamic	14.70	14.68-14.73
Propionic	15.50	15.48-15.52
Butyric	18.70	8.68-18.72
Hippuric	28.11	28.10-28.30

† Average of four replicates.

Table 2. The quantity of organic acids determined in four types of recombined cheese by HPLC

Organic acids	$\mu g\ g^{-1}$ cheese†			
	#1	#2	#3	#4
Oxalic	63.4	99.4	103.5	122.2
Orotic	23.0	18.0	22.8	20.6
Citric	993.0	1294.4	1184.8	727.2
Oxalacetic	11.1	2.9	30.1	21.7
Succinic	497.2	410.0	577.6	832.4
Lactic	6490.2	9812.3	9946.4	15953.3
Formic	836.1	646.7	791.5	646.7
Acetic	176.0	258.9	315.4	736.7
D-Pyroglutamic	21.5	49.3	19.5	50.7
Propionic	649.1	192.3	856.0	807.7
Butyric	774.9	410.3	833.7	821.9
Hippuric	-	-	13.0	-
Total	10505.7	13194.5	14649.3	20741.0

† Average of duplicate samples.

Table 3. The quantity of organic acids determined in four types of natural white brined cheese processed in the pilot plant or prepared from the market by HPLC.

Organic acids	μg g^{-1} cheese†			
	Pilot§	Rural	Industrial	Danish feta
Oxalic	65.9	Tr.¶	224.7	Tr.
Orotic	20.2	4.5	21.7	7.4
Citric	Tr.	262.2	1182.9	2272.6
Oxalacetic	Tr.	Tr.	110.3	72.5
Succinic	319.1	316.6	107.3	623.9
Lactic	15867.7	16415.9	18726.6	23298.9
Formic	-	Tr.	Tr.	Tr.
Acetic	716.5	579.7	1063.3	348.5
D-Pyroglutamic	50.9	17.9	228.1	219.2
Propionic	933.5	6904.6	1776.0	2000.0
Butyric	408.4	257.4	274.7	315.6
Hippuric	-	Tr.	Tr.	Tr.
Total	18382.2	24758.8	24682.0	29158.6

† Average of duplicate samples.

§ Cheese sample prepared from natural milk (3% fat) and ripened for two months.

¶ Tr. = Trace amounts.

The percent recoveries of organic acids (Table 5) revealed that the formic acid was the least recovered, 91.3%, from cheese samples. This table shows that percent recoveries of oxalic and oxalacetic acids were both 95%. Figs. 4 and 5 represent the standard curves for organic acids determined in this experiment. All related HPLC analyses revealed very high correlation coefficients (r=0.998-0.999) for peak area versus concentrations of standard acids.

CONCLUSIONS

The results of this study revealed that the method of extraction of organic acids from cheese samples and analysis with the HPLC system is very precise and significantly correlated with the respective standard curves. Variation of organic acids in different types of natural or recombined cheese is due to different processing or ripening conditions. This is related to the extent of biochemical activities of added enzymes or

lactic acid bacteria and also parameters such as temperature and brine concentration during the period of ripening.

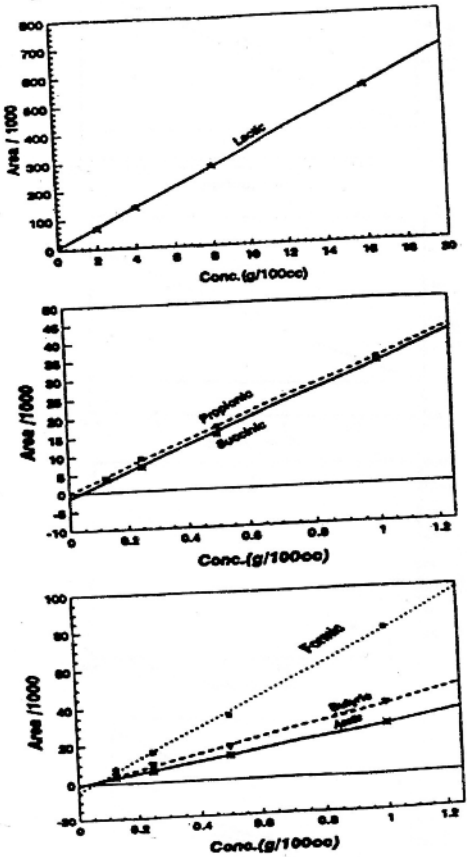

Fig. 4. Standard curves (peak area vs. concentration) of organic acids (>99.5%): lactic, propionic, succinic, formic, butyric, and acetic acids, determined by HPLC.

Table 4. Percent moisture content of recombined or natural cheese produced in pilot plant or purchased from market.

% Moisture†							
Recombined				Industrial	Pilot	Danish	Rural
#1	#2	#3	#4				
65.0	65.1	54.4	62.2	52.9	59.5	60.0	62.0

† Average of duplicate samples.

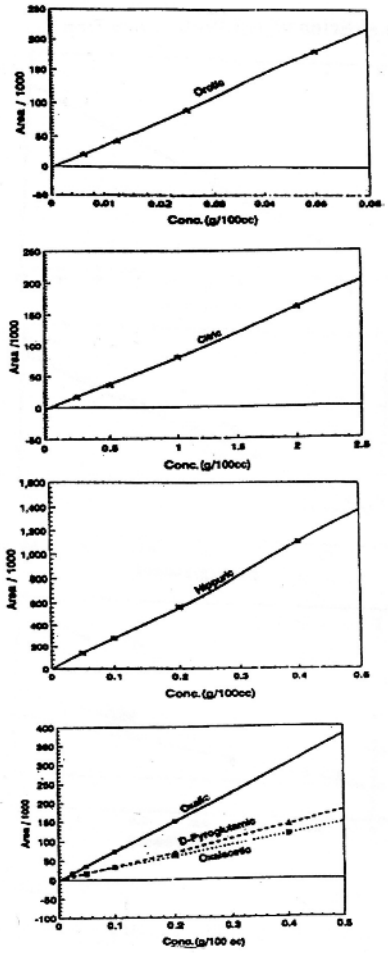

Fig. 5. Standard curves (peak area vs. concentration) of organic acids (>99.5%): orotic, citric, hippuric, oxalic, D-pyroglutamic, and oxalacetic acids determined by HPLC.

ACKNOWLEDGEMENT

The author wishes to thank the Research Council of Isfahan University of Technology for financial supports. Technical assistance of

Mr. B. Bahrami and Mr. R.A. Raddanipour through the whole research program at the Food Science and Technology Dept. laboratories and pilot plant is also appreciated. Thanks to Mrs. R. Dehghani for typing this manuscript.

Table 5. Percent recoveries of organic acids from cheese samples by HPLC†.

Organic acid	A	B	C	D	E
Oxalic	100	70	165	95	95.0
Orotic	500	26	550	524	104.8
Citric	1370	1100	2553	1453	106.0
Oxalacetic	1000	90	1060	950	95.0
Succinic	1030	572	1619	1047	101.6
Lactic	11000	18481	29333	10852	98.8
Formic	61000	0	55700	55700	91.3
Acetic	52500	396	57260	56891	108.3
D-Pyroglutamic	1000	248	1276	1028	102.8
Propionic	49500	1684	52351	50667	102.3
Butyric	48000	1486	48381	47000	97.9

† Average of duplicate samples.

A= µg organic acid added to one g of cheese.

B= µg organic acid present in one g of cheese.

C= µg total organic acid analyzed.

D= µg organic acid recovered.

E= Percent recovery of organic acid.

LITERATURE CITED

1. Blanco Gomis, D. 1992. HPLC analysis of organic acids. In: M.L. Leo (ed.). Food Analysis by HPLC. Marcel Dekker Inc. New York, U.S.A. 371-358.
2. Bushway, R.J., L.L. Bureau and D.F. McGan. 1984. Determination of organic acids in potatoes by high performance liquid chromatography. J. Food Sci. 49:75-81.
3. Dokhani, S. 1993. The study of processing and several physico-chemical properties of Iranian fermented white cheese from recombined milk. Iran. J. Agric. Sci. 24:27-38 (in Farsi).
4. Dokhani, S. and S. Malek. 1992. The study of processing and use of recombined milk for enzymatic coagulation in Iranian or foreign cheese

making. Presented at the 5th Cong. Food Sci. Tech. Iran, Abst. p.22 (in Farsi).

5. Homewood, J.T. 1986. Technology for the developing countries. In: R.K. Robinson (ed.). Modern Dairy Technology, Elsevier Applied Science Publisher, New York, U.S.A. 381-429.
6. Houlberg, U. 1988. HPLC of acids and carbohydrates in cultures.GA-37-CHR Hansens Laboratorium A/S Bullet., Copenhagen, Denmark. 3 p.
7. Lee, H.S. 1993. HPLC methods for separation and determination of nonvolatile organic acids in orange juice. J. Agric. Food Chem. 41: 1991-1993.
8. Malek, S. and S. Dokhani. 1992. Feta cheese production from recombined milk corrected with suitable minerals and milk proteins for better physico-chemical properties. Workshop Paper. Food Sci. and Tech. Dept., College of Agric., IUT, Iran 25 p. (in Farsi).
9. Marsilli, R.T., H. Ostapenko, R.E. Simons and D.E. Green. 1981. High performance liquid chromatographic determination of organic acids in dairy products. J. Food Sci. 46:52-57.
10. McFeeters, F.F. 1993. Single injection HPLC analysis of acids, sugars and alcohols in cucumber fermentation. J. Agric. Food Chem. 41: 1439-1443.
11. Mietton, B. 1988. Use of conventional processes for the manufacture of recombined cheese. IDF special issue. No. 9001, 275-289.
12. Palmer, J.K. and D.M. List. 1973. Determination of organic acids in foods by liquid chromatography. J. Agric. Food Chem. 21:903-906.
13. Picha. D.H. 1985. Organic acid determination in sweet potato by HPLC. J. Agric. Food Chem. 33:743-754.
14. Richardson, T. 1978. The hypocholesteremic effect of milk. A review. J. Food Protection. 41: 226-235.
15. Shimadzu, C.A. 1997. High performance columns for HPLC. Shimadzu Corporation International Marketing Division Catalogue, Kyoto-Tokyo, Japan. 85 p.
16. Sorensen, T.B. 1990. Dairy Technology. APV Pasilac AS Publisher. Aahus, Denmark. 36 p.
17. Wang, T., A.R. Gonzales, E.E. Gbur and J.M. Aselage.1993. Organic acid changes during ripening process of peaches. J. Food Sci. 58: 631-632.