

# Determination of Organic Acids, Sugars, Diacetyl, and Acetoin in Cheese by High-Performance Liquid Chromatography

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Sugars (lactose, glucose, and galactose), nonvolatile acids (citric, orotic, pyruvic, lactic, oxalic, and hippuric), some free fatty acids (formic, acetic, propionic, butyric, isobutyric, valeric, and isovaleric), diacetyl, and acetoin were separated on an Aminex HPX-87H column using a simple isocratic HPLC method and identified by retention times with ultraviolet and refractive index detectors. With the proposed technique it is possible to evaluate the development of microbial fermentations and, at the same time, degree of cheese ripening.

**Keywords:** Cheese; organic acids; sugars; diacetyl; acetoin; HPLC

## INTRODUCTION

For cheese ripening the evolution of the sugars and organic acids is very important. In fact, they directly or indirectly determine the chemical composition, as well as the sensory characteristics, and hence the quality.

To achieve a better separation of the main organic acids (citric, orotic, pyruvic, lactic, uric, formic, acetic, propionic, butyric, and hippuric) and sugars from dairy products, various HPLC methods have been reported (1–17). Determination of these compounds in dairy products always includes two phases: extraction from matrix and determination by HPLC.

Extraction of organic acids and sugars is a crucial step, but, according to Gomis (18), these substances (because of their high water solubility) could be extracted by blending the samples in water, acidified water, or a water solution of ethanol or acetonitrile.

A simplified method using only water at 50 °C as extracting agent was used by Mullin and Emmons (14). Marsili et al. (1) utilized acetonitrile and water (4:1) for extracting the organic acids from 5 g of cheese, but the same author four years later used a solution of barium hydroxide and zinc sulfate according to Dulley (4, 19). The above-mentioned barium hydroxide/zinc sulfate method was also used by McGregor and White (8). Panari (5) utilized water for the extraction, but the extract was applied to an anionic exchange resin (Amberlite CG 400) and the acids eluted with H<sub>2</sub>SO<sub>4</sub> 0.1N. For the determination of only the free fatty acids a steam distillation method has been proposed (15).

Many authors use the mobile phase of the following HPLC system (6, 7, 9–13, 17) for the extraction. Generally these methods used 0.0049–0.009 N of H<sub>2</sub>SO<sub>4</sub> as the extraction agent. Only Bevilacqua and Califano (6) used a solution of (NH<sub>4</sub>)<sub>2</sub>HPO<sub>4</sub> and acetonitrile. An interesting evaluation of the most used of these methods has been conducted by Lues et al. (16).

Also, the determination by HPLC is a crucial step in the analysis of sugars and organic acids in dairy products. Separation was done with the ion-exchange column (5, 9, 11, 13–17) or reversed-phase column (6).

Generally the acids were determined with an UV detector or a diode array set at 210, 214, 220, or 285 nm. For the sugars, a differential refractometer or a pulsed amperometric detector determination was used.

Simultaneous determination of acids and sugars has been reported by a few authors (9, 11, 14) but most have reported analytical problems for the coelution of citric and orotic acids, of lactose and citric acids, and of uric and formic acids. A partial solution of these problems was reported by González de Llano et al. (13), but they quantified only the organic acids.

Currently, a method that allows simultaneous quantification of acids, sugars, diacetyl, and acetoin in dairy products does not exist. The aim of this study, then, was to develop a simple and fast method for the extraction of these compounds from cheese and other dairy products and their determination with a simple isocratic HPLC analysis.

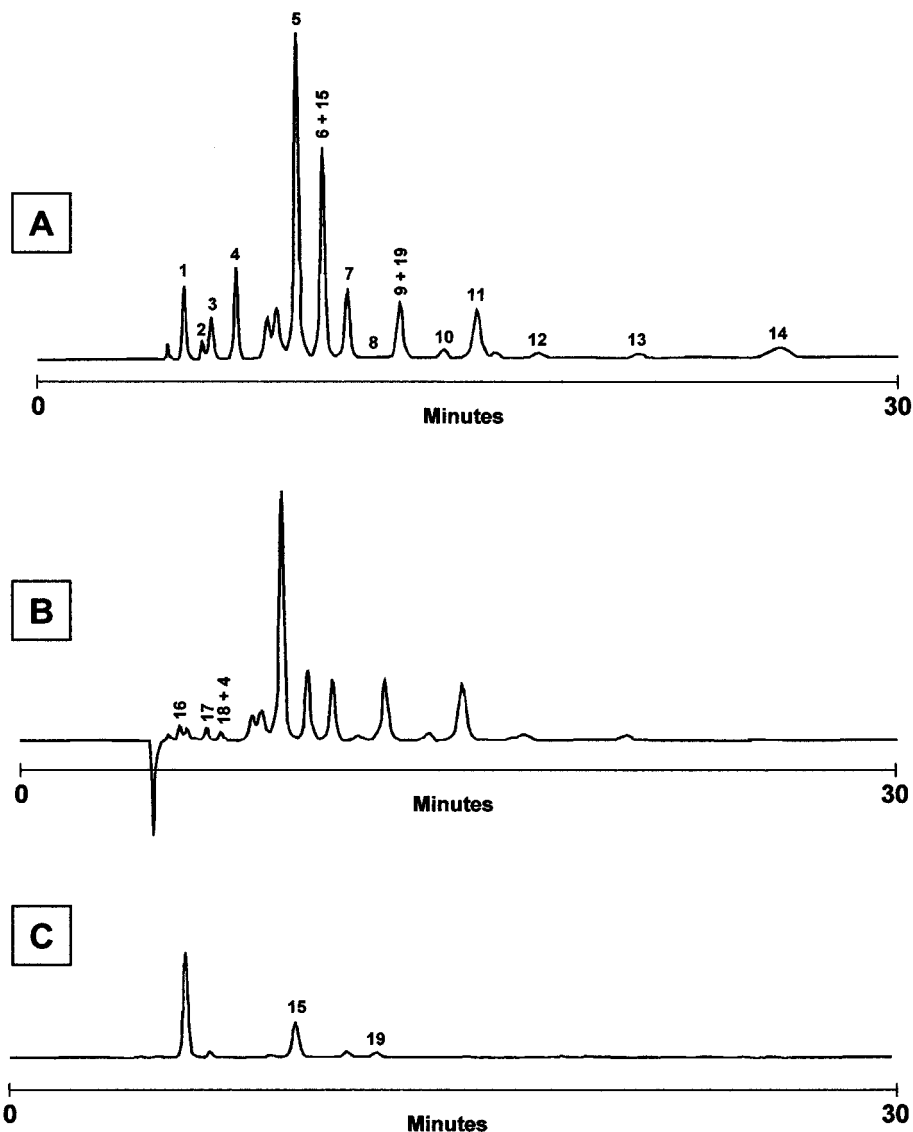
## MATERIALS AND METHODS

**Equipment and Operating Conditions.** The HPLC system (Thermo Quest, San Jose, CA) was equipped with an isocratic pump (P1000), a multiple autosampler (AS3000) fitted with a 20- $\mu$ L loop, a UV detector (UV100) set to 210 and 290 nm, and a refractive index detector (RefractoMonitor IV). The detectors were connected in series. Data were collected on a EZChrom ver. 6.6 system (Thermo Quest, San Jose, CA).

The analyses were performed isocratically at 0.8 mL/min and 65 °C with a 300  $\times$  7.8 mm i.d. cation exchange column (Aminex HPX-87H) equipped with a cation H<sup>+</sup> microguard cartridge (Bio-Rad Laboratories, Hercules, CA). Mobile phase was 0.013 N H<sub>2</sub>SO<sub>4</sub> prepared by diluting reagent grade sulfuric acid with distilled water, filtering through a 0.45- $\mu$ m membrane filter (Sartorius, AG, Göttingen, Germany), and degassing under vacuum. Analytical grade reagents were used as standards (Sigma Chemical, Milano, Italy).

**Sample Preparation.** For evaluating the method, 15 samples of Ossolano cheese were used. The analyzed samples, ripened for 40 days, were produced by farmhouses from raw

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**Figure 1.** HPLC separation of organic acids (1, ossalic; 2, citric; 3, orotic; 4, piruvic; 5, lactic; 6, formic; 7, acetic; 9, propionic; 10, isobutyric; 11, butyric; 12, isovaleric; 13, valeric; 14, hippuric; and 15, uric), sugars (16, lactose; 17, glucose; and 18, galactose), diacetyl (8), and acetoin (19) for an aqueous standard solution with UV detector set at 210 nm (A), 290 nm (C), and refractive index (B).

milk and purchased from specialist cheese shops. The samples were stored in sealed packets in the dark at 4 °C until required for analysis.

According to the results of Lues et al. (16), samples were prepared using a modification of the method of Bouzas et al. (9) because extraction with a sulfuric acid solution is more suitable for extracting organic acids. Cheese (5 g) was added to 25 mL of 0.013 N H<sub>2</sub>SO<sub>4</sub> (mobile phase) whereafter they were extracted for 10 min with a Stomacher (PBI, Milano, Italy). The use of a Stomacher allowed a reduction in extraction time because the Stomacher extracting is considerably more effective than a magnetic stirrer. The extract was subsequently centrifuged for 5 min (7000g) and the supernatant was filtered through a 0.20- $\mu$ m disposable syringe membrane filter (Sartorius AG, Göttingen, Germany).

**Calibration and Calculations.** Repeatability of the method was determined by calculating the standard deviations ( $\sigma$ ) of the analytes concentration between 10 consecutive HPLC runs of a solution obtained by diluting 2 times a concentrated standard solution (mother solution) (Table 1).

The method linearity was determined with six aqueous standard solutions for each compound. These solutions were obtained by diluting the mother solution 0, 2, 5, 10, 20, and 25 times. Three injections were made for each solution. For

**Table 1. Concentrations of Standard Solutions Used for Method Evaluation**

standard	concentration (g/L)
ossalic acid	0.381
lactose	0.882
citric acid	0.402
orotic acid	0.021
glucose	0.852
piruvic acid	0.493
galactose	1.523
lactic acid	24.174
uric acid	0.322
formic acid	8.518
acetic acid	6.104
diacetyl	0.872
propionic acid	5.824
acetoin	1.092
isobutyric acid	1.872
butyric acid	6.216
isovaleric acid	1.882
valeric acid	1.918
hippuric acid	0.017

each compound the linear regression coefficient between theoretical and measured concentrations was calculated.

The same calibration curve was used for determining the detection limits. This was established as the concentration showing a signal value at twice the noise.

The efficiency of the extraction procedure was evaluated by determination of recovery from a spiked cheese preparation. A known amount of standard solution obtained by diluting the mother solution 10 times was added to a 5 g sample of cheese and extracted as described above. Three injections were made for a sample of cheese and cheese plus added compounds.

Finally, quantification of analytes in cheese samples was based on the external standard method. Factor responses were calculated by three injections of the mother solution.

## RESULTS AND DISCUSSION

To improve the ion-exchange methods previously described, different operating conditions were assayed.

To achieve accuracy, according to Panari (5), sulfuric acid 0.013 N in the mobile phase was chosen. The eluent flow was set to 0.8 mL/min and the column temperature was set to 65 °C. With these operating conditions the analysis time is only 30 min and the pressure in the system is very low (60 psi).

With this column and H<sub>2</sub>SO<sub>4</sub> 0.013 N as mobile phase, the peak resolution is better and it is possible to directly determine sugars, acids, diacetyl, and acetoin with an UV detector and a RI detector coupling (9, 11).

Citric and orotic acid are resolved, unlike Bouzas et al. (11), but there is a coelution of galactose and piruvic acid, acetoin and propionic acid, and uric and formic acids.

To achieve the evaluation of these substances the best absorbance wavelength was determined after obtaining the individual absorbance spectra on a Shimadzu double-beam UV-1601 digital spectrophotometer.

The highest absorbance peak for acetoin and piruvic, propionic, uric, and formic acids is at 210 nm, whereas for acetoin and uric acid the highest absorbance peak is at 290 nm. At these wavelengths, the galactose shows no absorbance peaks, and so by using both the UV detector with two different wavelengths together with the RI detector the simultaneous evaluation of these substances is possible.

Typical chromatograms of a standard solution are shown in Figure 1: complete separation was achieved for the compounds under study.

To determine the concentrations of the coeluting compounds the following calculations were applied.

Piruvic acid:

peak height  
(or area) at 210 nm/response factor at 210 nm

Acetoin:

peak height  
(or area) at 290 nm/response factor at 290 nm

Uric acid:

peak height  
(or area) at 290 nm/response factor at 290 nm

Galactose:

$$[PH_{RI} - (C_{PA} \times RF_{PARI})]/RF_{GRI}$$

Where  $PH_{RI}$  = Peak height (or area) of mix at refractive index,  $C_{PA}$  = concentration of piruvic acid evaluated at 210 nm,  $RF_{PARI}$  = response factor for piruvic acid at

**Table 2. Retention Times (RT), Capacity Factors (K), Standard Deviation ( $\sigma$ ), and Coefficient of Variation (CV%) Calculated with a Standard Solution for Each Compound Determined<sup>a</sup> (10 Injections)**

compound	RT	K'	$\sigma$	CV%
ossalic acid	5.05	0.12	0.011	5.9
lactose	5.53	0.23	0.017	4.0
citric acid	5.68	0.26	0.013	6.7
orotic acid	5.97	0.33	0.00083	7.9
glucose	6.33	0.41	0.02	4.8
piruvic acid	6.83	0.52	0.015	6.3
galactose	6.92	0.54	0.081	10.7
lactic acid	8.89	0.98	0.435	3.6
uric acid	9.72	1.16	0.0095	5.9
formic acid	9.77	1.17	0.166	3.9
acetic acid	10.62	1.36	0.183	6.0
diacetyl	11.48	1.55	0.0187	4.3
propionic acid	12.41	1.76	0.172	5.9
acetoin	12.5	1.78	0.041	7.5
isobutyric acid	13.9	2.09	0.037	4.0
butyric acid	14.71	2.27	0.155	5.0
isovaleric acid	17.15	2.81	0.045	4.8
valeric acid	20.57	3.57	0.045	4.7
hippuric acid	25.31	4.62	0.00051	6.0

<sup>a</sup>  $K' = (t_r - t_m)/t_m$  where  $t_r$  = retention time of compound;  $t_m$  = retention time of an unretained component.

refractive index, and  $RF_{GRI}$  = response factor for galactose at refractive index.

Propionic acid:

$$[PH_{210} - (C_A \times RF_{A210})]/RF_{PA210}$$

Where  $PH_{210}$  = peak height (or area) of mix at 210 nm,  $C_A$  = concentration of acetoin evaluated at 290 nm,  $RF_{A290}$  = response factor for acetoin at 210 nm, and  $RF_{PA210}$  = response factor for piruvic acid at 210 nm.

Formic acid:

$$[PH_{210} - (C_{UA} \times RF_{UA210})]/RF_{FA210}$$

Where  $PH_{210}$  = peak height (or area) of mix at 210 nm,  $C_{UA}$  = concentration of uric acid evaluated at 290 nm,  $RF_{UA290}$  = response factor for uric acid at 210 nm, and  $RF_{FA210}$  = response factor for formic acid at 210 nm.

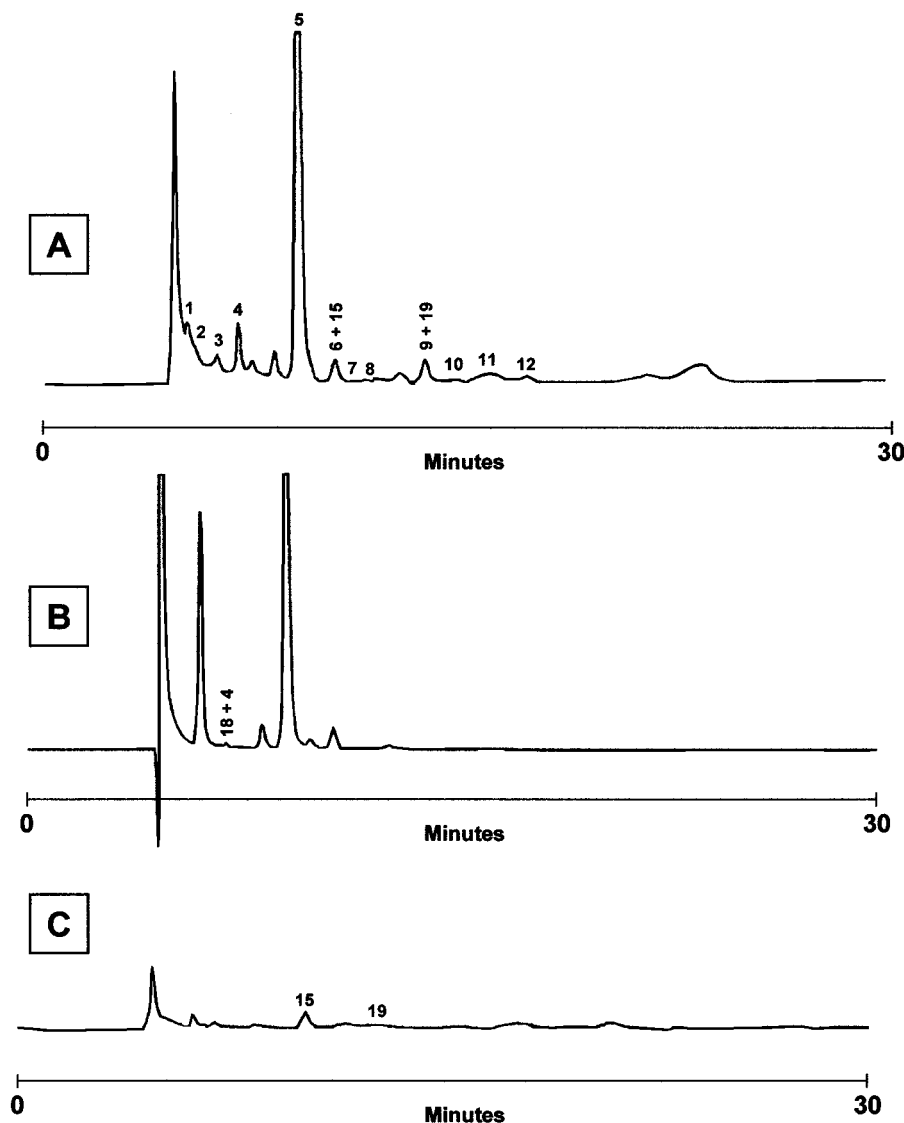
The coefficient of variation (CV%) for most of the tested compounds (Table 2) was lower than 8% except for galactose (10.7%), indicating good reproducibility. St-Gelais et al. (11) also found the coefficient of variation for galactose higher than 10%.

In Table 3 the linear regression coefficients calculated for the different compounds are reported. Although the concentration range used for the determination of the linearity is very wide, the regression coefficients are between 0.9914 and 0.9996.

Moreover, the detection limits of the method can be seen in Table 3. They vary from 0.22 mg/L for orotic acid to 119 mg/L for galactose. Because of the greater noise of the RI detector compared to the UV detector the sugar limit is higher.

The recovery of analyzed compounds added to cheese is high except for lactose and orotic acid (Table 4) in agreement with Bevilacqua and Califano (6), but only slightly inferior to those indicated by other authors (1, 11).

By comparison with solvent peaks of the standard solutions, the solvent peak in the sample cheese chromatogram is very extensive, occurring between 4 and 6 min and resulting from various unretained components



**Figure 2.** HPLC separation of organic acids (1, ossalic; 2, citric; 3, orotic; 4, piruvic; 5, lactic; 6, formic; 7, acetic; 9, propionic; 10, isobutyric; 11, butyric; 12, isovaleric; 13, valeric; 14, hippuric; and 15, uric), sugars (16, lactose; 17, glucose; and 18, galactose), diacetyl (8), and acetoin (19) for a typical commercial cheese with UV detector set at 210 nm (A), 290 nm (C), and refractive index (B).

**Table 3. Linear Regression Coefficients ( $R^2$ ) and Detection Limits (g/Kg) for Described Method**

compound	$R^2$	detection limit
ossalic acid	0.9970	0.018
lactose	0.9951	0.323
citric acid	0.9985	0.0077
orotic acid	0.9914	0.00014
glucose	0.9919	0.296
piruvic acid	0.9976	0.0016
galactose	0.9949	0.595
lactic acid	0.9993	0.018
uric acid	0.9982	0.0019
formic acid	0.9983	0.0089
acetic acid	0.9974	0.018
diacetyl	0.9709	0.286
propionic acid	0.9980	0.0196
acetoin	0.9969	0.130
isobutyric acid	0.9966	0.036
butyric acid	0.9996	0.019
isovaleric acid	0.9985	0.048
valeric acid	0.9977	0.059
hippuric acid	0.9974	0.00015

**Table 4. Recovery (%) and Coefficient of Variation (CV%) for Each Compound Determined (three injections)**

compound	recovery (%)	CV%
ossalic acid	92.6	19.3
lactose	80.3	8.2
citric acid	92.5	11.0
orotic acid	80.8	7.1
glucose	90.1	5.8
piruvic acid	91.9	4.8
galactose	85.7	4.5
lactic acid	88.6	6.7
uric acid	87.2	5.9
formic acid	89.4	7.3
acetic acid	84.6	5.2
diacetyl	88.6	9.2
propionic acid	89.8	6.1
acetoin	90.1	7.4
isobutyric acid	89.3	10.7
butyric acid	88.9	11.9
isovaleric acid	94.9	4.7
valeric acid	85.5	4.9
hippuric acid	93.8	10.4

(Figure 2). Integration of near peaks is difficult for the occurrence of this solvent peak and therefore there is wider variability, particularly for citric acid (Table 4).

Checking the Ossolano data, lactic acid is the main acid of this cheese and its high concentration could be attributed to the long ripening period (Table 5). The



**Table 5. Mean Concentration ( $\bar{X}$ ) Expressed as g/kg and Standard Deviation ( $\sigma$ ) for Each Compound Determined in the 15 Samples of Ossolano Cheese (three injections per sample)**

compound	$\bar{X}$ (g/kg)	$\sigma$
ossallic acid	0.06	0.07
lactose	0.87	0.02
citric acid	0.38	0.44
orotic acid	0.04	0.01
glucose	0.42	0.35
piruvic acid	0.18	0.15
galactose	1.01	1.64
lactic acid	62.01	12.19
uric acid	0.70	0.50
formic acid	2.96	1.06
acetic acid	2.96	0.81
diacetyl	4.52	2.54
propionic acid	1.2	0.66
acetoin	0.17	0.12
isobutyric acid	nd <sup>a</sup>	
butyric acid	2.25	1.43
isovaleric acid	0.78	0.04
valeric acid	nd	
hippuric acid	nd	

<sup>a</sup> nd, not determined.

amount of citric acid used like a carbon source is very low. On the contrary, the acetic, propionic, and butyric concentrations due to butyric and propionic acid fermentation are high. The diacetyl concentration is very high because the Ossolano cheese is produced with whole milk. The concentration of the acids averages between those of a Fontina and a Parmigiano Reggiano (5).

The described procedure seemed well-suited for analysis of various sugars and organic acids in dairy products. The main advantages of this method are (1) the simple sample preparation (a single extraction); (2) low costs of extraction solvent and mobile phase; (3) low environmental damage because only a small concentration of sulfuric acid is used for the extracting agent and mobile phase; (4) chromatographic time of only 30 min per sample; (5) a linear response for a wide concentration range; (6) a coefficient of variation lower than 8% for all compounds except for galactose; and (7) a recovery over 80%.

Also, the column is one that is commonly used, and no loss of column efficiency was observed during the course of this study.

This HPLC method is therefore fast and accurate, and differs from those previously suggested as it allows the simultaneous determination of acids, sugars, diacetyl, and acetoin using a very simple HPLC system existing at all research laboratories.

#### ACKNOWLEDGMENT

The authors thank A. Grosso for technical assistance.

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Received for review July 27, 2000. Revised manuscript received April 4, 2001. Accepted April 9, 2001. This work was supported by the Regione Piemonte–Interreg II Switzerland-Italy Project.

JF0009403