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CONCENTRATIONS OF CITRATE AND KETONE BODIES IN COW'S RAW MILK

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Abstract

The milk composition of high-yielding dairy cows is highly related to their nutrition. The energy deficiency in dairy cows can cause subclinical or clinical ketosis. In ketotic state the concentration of ketone bodies (acetone, AC; acetoacetic acid, ACAC; and β-hydroxybutyric acid, BOHB) increases in the different body fluids and simultaneously, changes in concentration of other compounds are also expected. The authors' hypothesis is that the members of the TCA-cycle, such as citric acid (CA) also change in association of increased formation of ketone body. To support this hypothesis the concentrations of ketone body components and CA were parallel measured and their relationship was studied in raw milk samples.

Based on the AC concentration of the milk samples two groups were formed retrospectively: High Acetone (HA) Group ($n = 41$) of AC concentration > 0.4mM and Low Acetone (LA) Group ($n = 78$) with the AC level ≤ 0.4 mM.

In all samples very low ACAC level was found, which is a consequence of the spontaneous decarboxylation of ACAC to AC during the usual sample storage. Focusing on the results of HA Group the authors found significant relationship between CA and ketone bodies and a parallel drop of AC and CA during the metabolically crucial first 1–4 weeks of lactation. For this reason they suggest to introduce simple, easy, automated methods to determine AC and/or CA concentration in raw milk.

Keywords: milk, ketone bodies, acetone (AC), acetoacetic acid (ACAC), β-hydroxybutyric acid (BOHB), citric acid (CA), flow injection analysis (FIA), gas chromatography.

1. Introduction

In various disorders of energy metabolism the concentrations of milk constituents also change. In healthy high-yielding cows the inadequate energy intake during the late pregnancy and early lactation leads to stronger ketogenesis, in which the increasing level of ketone bodies (acetone, AC; acetoacetic acid, ACAC; and β-hydroxybutyric acid, BOHB) in body fluids results ketonaemia, ketonuria and in lactating animals ketolactia. These conditions are associated with decreased milk yield, reproductive performance and increased risk of clinical ketosis. The early detection of elevated levels of ketone bodies – mainly during the subclinical stage of energy deficiency – is highly recommended. On this basis appropriate corrections

can be carried out in the herd management to reduce the profit loss in milk production and the risk of clinical ketosis [1], [2], [3].

To reveal the energy deficiency and/or diagnose ketosis in field conditions only test strips or other simple reagent kits are used in blood or urine samples, while raw milk is the most easily available biological fluid in milking cows [1], [2]. In spite of the fact that laboratory analysis for ketone bodies is more sensitive than field tests, milk samples are rarely sent to laboratory only for ketone body determination, while the milk is regularly analysed in laboratories for its other constituents (protein, fat, lactose, urea, cell count, etc.) during lactation. Therefore it is obvious that these regularly investigated milk samples could be analysed for ketone bodies as well using a sensitive and standardized method. Measurement of milk ketones is anyhow a useful tool in early evaluation of subclinical/clinical ketosis [1], [4].

In addition to elevated ketogenesis, further metabolic changes such as decreased amount of tricarbonic acid cycle (TCA-cycle) intermediates might be expected in the extracellular fluids during energy deficiency. The citric acid (CA) is an important member of TCA-cycle and it has regulatory effect on acetyl-CoA metabolism in liver mitochondria [5]. Its amount in cow's body fluids varies with many diseases. Amount of CA in cow's milk is 10 mM [6]. Many papers have reported so far that in healthy cows the CA content of milk was higher in the early stages of lactation and its concentration gradually decreased as lactation advanced [7], [8]. It is interesting that this phenomenon has not been studied in detail so far. In pathological conditions, such as subclinical mastitis the milk CA decreases proportionately to the degree of inflammation [9].

The effect of stage of lactation on presence and distribution of ketone bodies and on concentration of CA in cow's milk has not been studied. Similarly, no papers are available clarifying that correlation between ketone bodies and CA in milk exists or not.

We suppose that the concentration of CA in raw milk changes after calving, especially within the energetically most critical first few weeks of lactation. It is also assumed that a relationship exists between ketone body and CA level of the milk (*Fig. 1*). To prove these hypotheses two exact, precise and reproducible, but relatively sophisticated time- and/or money-consuming methods (gas chromatography and fluorimetry) and a rapid method (flow injection analysis, FIA) were used in milk samples of healthy, high-producing dairy cows during the early lactation period.

If our theories became true under real conditions, introducing an automated CA determination from raw milk could be an additional analytical tool to reveal or confirm energy deficiency.

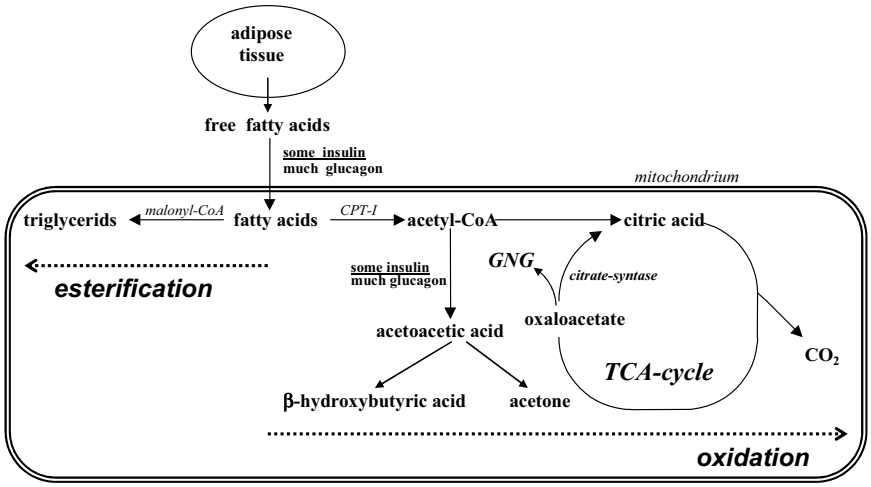

Fig. 1. Schematic connection, formation and transformation of ketone bodies related to TCA-cycle [16] (TCA-cycle=tricarbonic-acid-cycle, CPT-I=carnitine-palmitoil-transferase-I, GNG=gluconeogenesis)

2. Materials and Methods

2.1. Milk Samples

Samples under real circumstances of sampling, transport and storage were analysed. Milk samples of healthy Holstein-fries cows ($n = 119$) were collected randomly at the regular morning milkings at 7 dairy herds in Hungary 10–90 days after calving and sent for measuring their composition (protein, fat, lactose, urea, somatic cell count, etc.) to the Hungarian Herd Recording Ltd., Gödöllő, Hungary. The origin of the samples, and the day of the lactation was precisely recorded. An aliquot of all samples arrived at our department on the same day in a well-sealed, cooled (+4°C) 200 ml bottle. The milk samples were stored at −18 °C in the laboratory to prevent their further chemical changes, such as spontaneous decarboxylation of ACAC to AC. All chemical analyses were carried out within 48 hours after milking.

Following the laboratory analysis of milk samples two groups were formed retrospectively, on the basis of their AC concentrations. In the low acetone group (LA Group, $n = 78$) the milk acetone level was ≤ 0.4 mM whereas in the high acetone group (HA Group, $n = 41$) it was > 0.4 mM.

2.2. Reagents

Acetone and methylethylketone reference substances for gas chromatography were purchased from Merck, Germany (catalogue numbers: 100020 and 109709). All other chemicals were analytical grade obtained from Reanal Ltd., Hungary.

2.3. Gas Chromatographic Determination of Ketone Bodies

For determinations of oxidised, reduced and total ketone bodies from raw milk samples a headspace gas chromatographic (HS-GC) method including appropriate sample preparations was applied [10]. Sample preparation is based on chemical oxidation of ketone bodies to acetone in three consequent steps.

For analytical AC determinations a 10 mM AC stock solution was prepared and diluted in the range from 0.05 to 10 mM with distilled water. These AC standard solutions also contained the necessary internal standard (0.1 μl methylethylketone/ml).

The AC, ACAC and BOHB concentrations were calculated from three AC determinations.

The method was carried out with an adsorption chromatographic system. A Carlo Erba (Germany) Vega Series 2 GC model with 6 ft. column packed with Porapack Q 80/100 mesh (Waters Ltd., USA) and equipped with flame ionisation detector (Carlo Erba, Germany) was used. In order to protect the analytical column from contamination with the matrix substance of milk samples, a pre-column packed with glass wool was inserted in front of the analytical column.

Data were collected with an HP 35900 ADC interface and were analysed by an HP ChemStation software (Hewlett Packard Ltd., USA).

Analytical conditions:

Column temperature:	isotherm, 175 °C
Sampling:	controlled pressure headspace
Sample loop:	3 ml
Injector temperature:	250 °C
Internal standard:	0.1 μl methylethylketone/ml sample
Detector temperature:	250 °C
Carrier gas:	He

The ratio of each acetone peak area to the internal standard's peak area was determined and plotted against the concentration ratio.

2.4. Flow Injection Determination of AC

The developed and optimised method [10] is based on the relatively high volatility of the acetone content of the injected milk sample into the carrier solution, it can

be throughput on the gas diffusion membrane and so it appears in the reagent stream containing indicator and hydroxylamine. The acetone and hydroxylamine react to form acetoxyme. The pH change during the chemical reaction is detected photometrically. According to the principle of FIA method, a standard flow injection system (Enviroflow 5012 System and Detector System 5042 made by Foss-Tecator, Sweden) with gas diffusion unit (Chemifold V XS, Foss-Tecator, Sweden) and thermostat (FIAstar 5101, Foss-Tecator, Sweden) were applied.

Analytical conditions:

Sample volume:	200 μl
Carrier:	2 ml/min, phosphate buffer (0.1 M, pH = 7) containing 1.2 g 30% Brij 35
Reagent:	1.5 ml/min, 100 ml methylorange indicator stock solution (0.25 g/l) plus 150 ml hydroxylamine stock solution (20 g/l) were diluted with distilled water to 1000 ml
Detection:	540 nm
Cycle time:	80 sec
Reaction coil:	0.5 × 60 cm
Thermostat:	80 °C

2.5. Fluorimetric Determination of Citric Acid

A sensitive and specific fluorimetric method for determination of CA from raw milk samples [11] was applied with some modifications.

Before the analysis 4 ml milk was centrifuged in a clear 10 ml tube (10000/min, $g = 3100$) for 20 min to remove the colloidal particles. Distilled water of 0.95 ml and 1 ml metaphosphoric acid solution (2 w/v%) were added to 0.05 ml of milk sample supernatant or standard citric acid solution. The mixture was shaken and centrifuged (3000/min, $g = 950$) for 10 min. One ml of the supernatant was taken into a digestion tube and 2 ml of o-ATH (o-aminothiophenol, 0.75 w/v % in 50 w/v % phosphoric acid) solution was added. After replacement the air with nitrogen gas the test tube was closed and allowed to react at 125°C for 15 h in an air thermostat. The reaction mixture was cooled with water, then 3 g sodium-chloride and 4 ml ethylacetate was added, the test tube re-sealed and re-shaken for 5 min. The upper ethylacetate layer was separated and its fluorescence intensity was measured with excitation at 415 nm and emission at 450 nm by a Jasco FP 920 (Italy) standard fluorimeter.

2.6. Statistical Analysis

Results were analysed statistically with frequency analysis, correlation analysis, significance tests and regression analysis by Statistica 5.5 software (StatSoft Inc., USA).

3. Results

3.1. Ketone Bodies

The HS-GC and the FIA method developed for determination of AC were parallelly investigated to establish their performance parameters for determination of AC (*Table 1*). *Fig. 2* presents the HS-GC raw chromatograms for its calibration and *Table 2* contains the recovery efficiencies for individual ketone bodies.

Table 1. Analytical parameters of HS-GC and FIA methods for determination of acetone in milk

Investigated parameter	HS-GC	FIA
Limit of detection, mM	0.01	0.03
Linear range, mM	0–10	0–10
Limit of determination, mM	0.015	0.05
Calibration requirement	only control required	before each batch
Sensitivity (slope of the calibration graph)	0.777 (peak /conc. ratios)	225.9 (AU/mM)
Error of analytical determinations, RSD%	< 1	–
Reproducibility, RSD%	< 2.0	< 2.2
Precision (average recovery), %	100.51	103.82
Average retention time, min	5.14	–
Sample preparation	minimal	minimal

Table 2. Average recovery efficiencies for individual ketone bodies in milk (each result represents 3 independent HS-GC determinations)

Ketone bodies	Recovery efficiency (%)
Acetone, 0.5 mM	92.7 ± 6.14
Acetone, 4.0 mM	96.1 ± 4.36
Acetoacetate, 0.5 mM	97.3 ± 3.45
Acetoacetate, 4.0 mM	101.1 ± 2.17
β-hydroxybutyrate, 0.5 mM	97.6 ± 2.24
β-hydroxybutyrate, 4.0 mM	96.4 ± 3.70

Distribution of ketone bodies in raw milk 48 hours after sampling is shown in *Fig. 3*. In both groups very small concentration of milk ACAC (2–2.5% of total ketone body) was found. Obviously, it was the BOHB that showed opposite

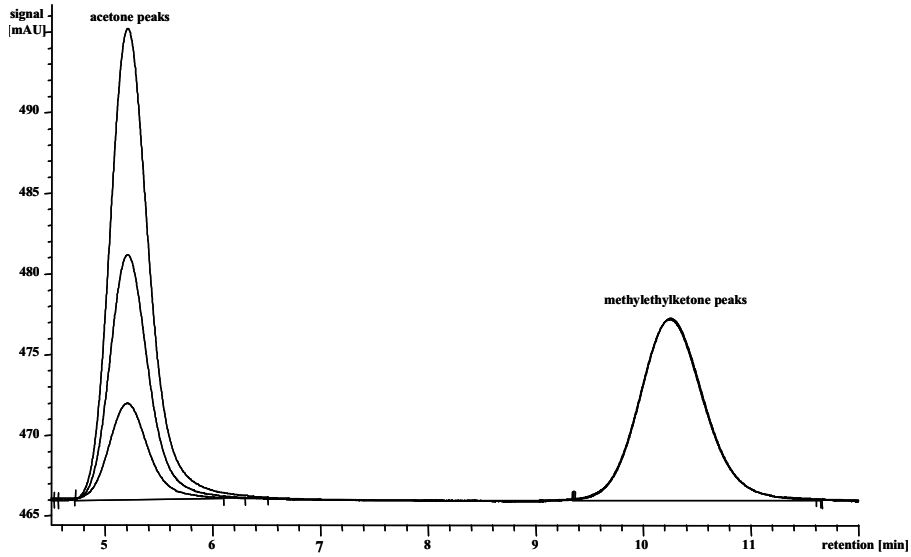

Fig. 2. Raw chromatograms for HS-GC calibration

distribution to AC in both groups: in the LA Group its ratio was 94.7% while in the HA Group it was only 16.3%.

Relationship among the investigated parameters is shown in *Table3*. In the LA Group low, but significant negative correlation ($r = -0.372$) was found between ACAC and BOHB. In this group milk AC did not correlate significantly with the other two ketone bodies. In the HA samples significant positive correlation and linear connection (BOHB $= 2.491 + 0.586 * \text{AC}$) existed only between AC and BOHB (*Table 3* and *Fig. 4*).

In *Fig. 5* ketone body and CA results are plotted against the days of lactation. The majority of high ketone body levels were measured mainly within the first six weeks of lactation, practically these samples belonged to the HA Group. Among ketone bodies the concentration of BOHB slowly elevated till the 60$^{\text{th}}$ day of lactation then its level became approximately constant. Changes in AC concentration showed an opposite tendency resulting in 80–90% drop of AC level by the 60$^{\text{th}}$ day after calving. ACAC concentration did not express any significant changes and remained very low in all samples along the whole 3-month period investigated.

3.2. Citric Acid

Similarly to the concentration of AC, highest but quickly and gradually decreasing concentration of milk CA was found in the first 4 weeks of lactation. CA reached its minimum level (approx. 2 mM) around in 40$^{\text{th}}$ day after calving (*Fig. 5*). After

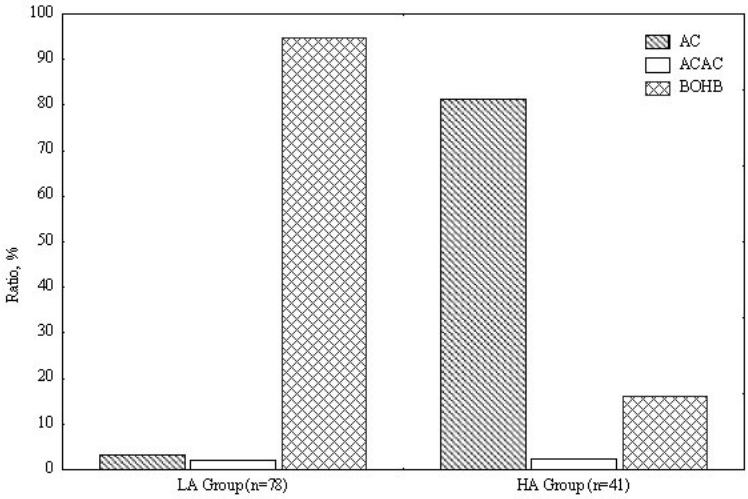

Fig. 3. Distribution of ketone bodies in raw milk, AC = acetone, ACAC = acetoacetic acid, BOHB = β-hydroxybutyric acid, CA = citric acid, LA = low acetone, HA = high acetone

Table 3. Correlations between ketone bodies and citric acid in raw milk, AC = acetone, ACAC = acetoacetic acid, BOHB = β-hydroxybutyric acid, CA = citric acid, LA = low acetone, HA = high acetone

	LA Group			HA Group		
	CA	AC	ACAC	CA	AC	ACAC
BOHB	-0.380^{xx}	0.066^{NS}	-0.372^{xx}	-0.579^{xxx}	0.623^{xxx}	0.164^{NS}
ACAC	0.002^{NS}	-0.208^{NS}		-0.170^{NS}	-0.157^{NS}	
AC	-0.081^{NS}			0.469^{xxx}		

^{x}: significant, $P < 0.05$; ^{xx}: $P < 0.01$; ^{xxx}: $P < 0.001$
^{NS}: not significant, $P \geq 0.05$

this minimum concentration of CA slightly increased, but even in the 3^{d} month its level was only around 30% of the basal value.

Correlations between ketone bodies and CA are shown also in *Table 3*. CA expressed negative correlation with BOHB in both groups and, which is more

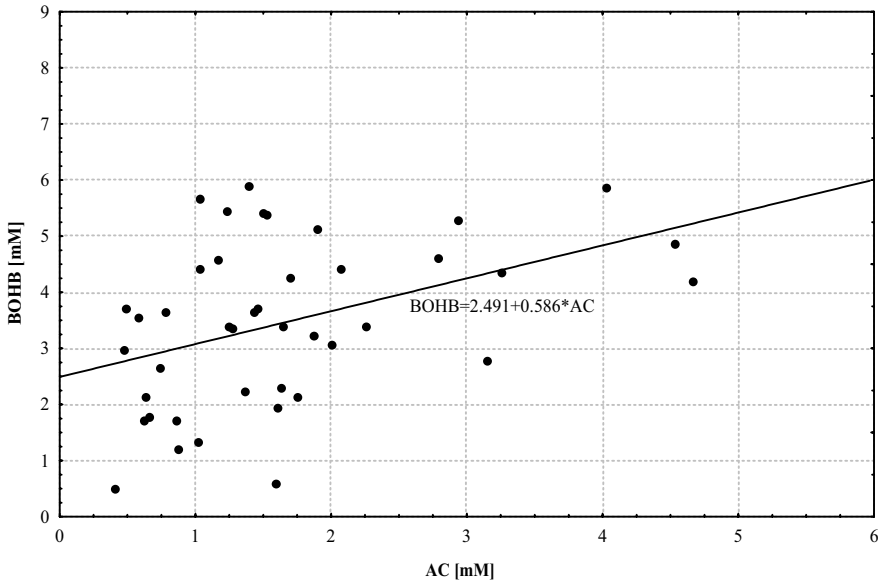

Fig. 4. Relationship between acetone and β-hydroxybutyric acid in HA milk samples, AC = acetone, BOHB = β-hydroxybutyric acid, HA = high acetone

important, positive correlation with AC in the HA group.

4. Discussion

The results obtained by optimised FIA method were compared with the results of the headspace sampling GC. From the investigations presented in *Table 1* the conclusion is: the FIA method can be a very good alternative to HS-GC in rapid cow's milk-monitoring. It is faster and more profitable especially considering costs per test.

Unfortunately, it is not clarified how the three ketone bodies are distributed in the milk of healthy and ketotic cows, most probably due to the absence of unified analytical methods. In blood serum of healthy mammalian species the following relative proportions are generally approved: 78% BOHB, 20% ACAC and 2% AC [12]. Exact data on relationship among the three ketone bodies in the raw milk is not available. In our study we observed a reverse distribution of ACAC and AC in milk compared to their distribution in blood published by SCHULTZ and MYERS [13]. At 4 °C or room temperature the sample's ACAC is unstable and disappears with a rate of 6%/day or 6%/hour, respectively [14]. This phenomenon most probably explains our observation that in the 48-hour-old milk samples only very low levels

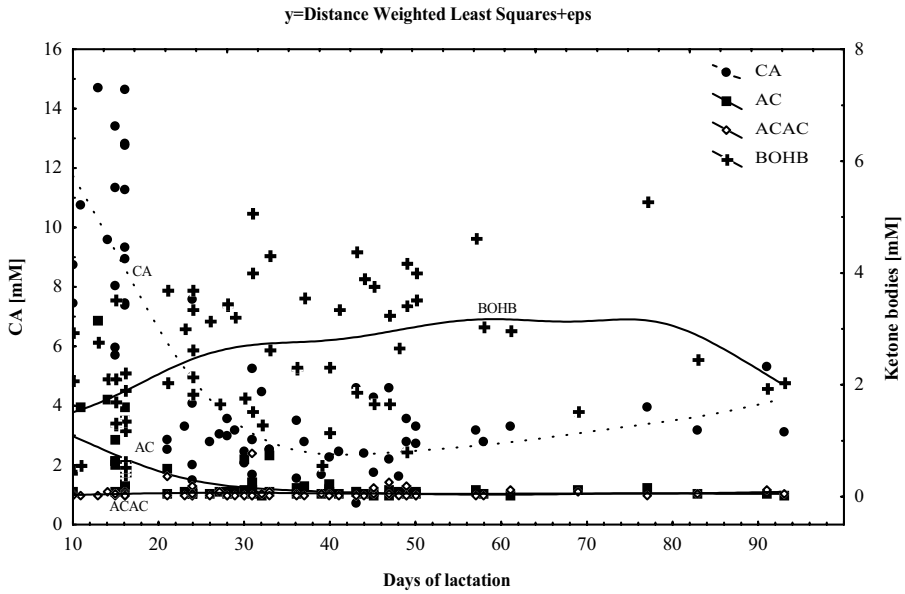

Fig. 5. Concentration of ketone bodies and citric acid in milk plotted against the days of lactation ($n = 119$), AC = acetone, ACAC = acetoacetic acid, BOHB = β-hydroxybutyric acid, CA = citric acid

of ACAC were found, but further investigations are needed.

The range of AC level in milk separating the normal and subclinical ketotic state in cows is claimed to be 0.4–0.7 mM [4]. On the basis of frequency analysis in our experiment we think the borderline is around 0.4 mM (*Fig. 6*).

The changes of milk CA found along the lactation meet the data of other authors [7], [8]. The relatively high CA concentration 10–20 days after calving can be explained by the intensive TCA-cycle this time. In spite of the fact that the milk production dramatically increases, immediately after calving it is lower than some weeks later at the peak lactation. The key factor in both ketogenesis and CA formation (TCA-cycle) is oxaloacetate: the lower the milk production, the more oxaloacetate is available to form CA with acetyl-CoA. Most probably, immediately after calving, other members of the TCA-cycle can also be found in higher concentration in the milk than 6–10 weeks after calving. Along the advanced lactation the activity of the TCA-cycle is supposed to be lower as this time oxaloacetate is used mainly for gluconeogenetic processes. Lack of oxaloacetate can explain the simultaneous decrease of CA and increase of keton bodies. There are some other milk constituents, such as milk protein, that are also decreasing with the advanced lactation [6] [15].

Nearly all of HA samples came from the early stage (first 2–4 weeks) of

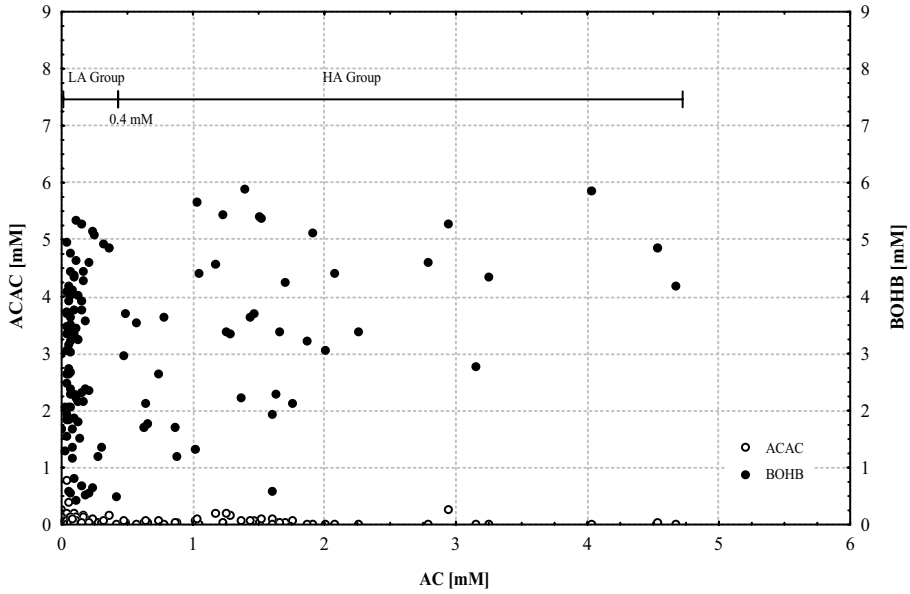

Fig. 6. Distribution of ketone bodies in all milk samples (frequency analysis), AC = acetone, ACAC = acetoacetic acid, BOHB = β-hydroxybutyric acid, CA = citric acid, LA = low acetone, HA = high acetone

lactation. As this period is critical in development of metabolic diseases associated with energy deficiency (fatty liver, ketosis), we focused on the results of the HA Group. The initial high level and the parallel drop of AC and CA in the milk samples of this group in the first few weeks of lactation are remarkable. We think that the regular, simultaneous control of their concentration in the milk could be used in monitoring the energy status of dairy cows. Determination of AC and that of CA can be easily automated via FIA and FTIR (Fourier transformation infrared analysis), respectively. Following the complete development of these simple, easy methods they should be introduced in the routine laboratory analysis of milk. This monitoring system does not require extra milk sampling, measurements can be carried out on milk samples monthly sent to milk laboratories for other purposes.

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