Fluorometric determination of free and total isocitrate in bovine milk

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ABSTRACT

Isocitrate is an intermediate metabolite in the citric acid cycle found both inside the mitochondria as well as outside in the cytosolic shunt. Oxidation of isocitrate is believed to deliver large fractions of energy [i.e., reducing equivalents (NADPH) in the bovine udder] used for fatty acid and cholesterol synthesis. This study describes a new analytical method for determination of free and total isocitrate in bovine milk where time-consuming pretreatment of the sample is not necessary. Methods for estimation of both total isocitrate and free isocitrate are described, the difference being the esterified or even lactonized isocitrate. On average, 20% (6–27%) of cow milk isocitrate was esterified and free isocitrate correlated significantly with total isocitrate ($r = 0.98$). The present fluorometric determination correlated well with the traditional spectrophotometric determination of isocitrate. Milk samples from Danish Holstein cows (984) contained significantly less isocitrate than milk from Danish Jersey cows (760; i.e., 0.134 vs. 0.211 mmol/L). Isocitrate in milk is correlated to milk protein, fat, and citrate, and it is speculated, based on biochemistry, former studies, and the present, that isocitrate may reflect the energy situation in the mammary gland. The use of isocitrate as a biomarker of the energy status in the dairy cow is warranted.

Key words: esterified isocitrate, milk, fluorometry, biomarker, energy status

INTRODUCTION

Isocitric acid is widespread in living material (e.g., bacteria, plant, and animal tissues). The formation of isocitrate is, in mammalian metabolism, closely connected to citric acid, which in turn is an important intermediate in energy metabolism. Isocitrate is oxidized by the enzyme isocitrate dehydrogenase (ICDH), where carbon dioxide is liberated and 2-oxoglutarate is the oxidized product. Three main groups of ICDH are known: (1) NAD⁺-dependent (EC 1.1.1.41), abundant

inside the mitochondria and an important regulatory region of the citrate cycle (TCA cycle); (2) NADP⁺-dependent form, also inside the mitochondria (EC 1.1.1.42, 2), and (3) NADP⁺-dependent form in the cytosol (EC 1.1.1.42, 1). However, both citrate and isocitrate are able to cross the mitochondrial membrane, alleviating intra- and intermitochondrial exchange of energy precursors.

Lipogenesis in the ruminant is very different from lipogenesis in nonruminant animals, both considering precursors for the FA and considering the origin of energy supply. In ruminants, about one half of the milk FA (molar percent) are derived from de novo synthesis (Bauman and Grinari, 2003). β -Hydroxybutyrate and acetic acid are important precursors in ruminant lipogenesis. Fatty acid synthesis is an energy-demanding series of reductive steps using reducing equivalents in the form of NADPH, namely 2 mol per 2C-incorporation. A significant part of the NADPH-production in ruminant mammary glands is believed to be oxidative decarboxylation of isocitrate (Bauman et al., 1970; Gumaa et al. 1973; Chaiyabutr et al., 1980). The latter process is mediated by extra mitochondrial ICDH (EC 1.1.1.42; 1), also believed to be the main energy source for synthesis of cholesterol (Moore and Christie, 1979). Cytosolic NADP⁺-dependent ICDH activity is therefore abundant in ruminant mammary cells (Bauman et al. 1970; Gumaa et al. 1973).

Some attention has been given to isocitrate and ICDH activity in ruminant mammary gland and milk to describe the physiological status of the animal or the production conditions. Most of these observations support the important role of citrate-isocitrate-2-oxoglutarate in the milk energy turnover.

Measurements of isocitrate in milk have so far all been based on spectrophotometry, which is not an ideal method considering the opaque protein and fat matrix. Analyses are performed after a pretreatment of the sample (i.e., centrifugation to separate the fat), followed by precipitation of protein with perchloric acid (HClO₄) to obtain a clear fraction and subsequent neutralization (Faulkner, 1980; Faulkner and Clapperton, 1981; Chaiyabutr et al., 1981; Faulkner et al., 1986).

The present analytical method for determination of isocitrate is principally based on the spectrophotometric

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(UV) method (Beutler, 1989); however, the produced NADPH is subsequently used in a second reaction for reduction of the nonfluorescent precursor resazurin to produce the fluorescent substance resorufin, which is detected by fluorometry. The present fluorometric technique renders pre-treatment of the sample superfluous. It is well recognized from food analyses that a certain fraction of the isocitrate may be esterified or lactonized (internal esterification; Boehringer Mannheim, 1995). We have used this obtained knowledge to distinguish between free and total isocitrate by the fluorometric method.

MATERIALS AND METHODS

Samples and Standard Analyses

Milk samples ($n = 1,744$) were from the resident herd at the Danish Cattle Research Centre (Tjele, Denmark). Analyzed samples were used to investigate the correlation among free isocitrate, selected milking data, other milk components, and udder health as indicated by milk SCC. The milking system is a robotic system, where representative milk samples are taken in a 10-mL tube, predosed with the preservative Bronopol (Myacide, Pharma BP, BSF Ltd., Nottingham, UK) to obtain a 100-mg/kg sample. The samples were stored at 4°C and brought to the laboratory every morning. Milk citrate, lactose, fat, and protein were determined by infrared spectroscopy (CombiFoss 4000, Foss Electric Ltd., Hillerød, Denmark). Determination of SCC was performed at a commercial laboratory (Eurofins, Holstebro, Denmark) using standard Fossomatic cell counter (EN ISO 13366-3; Foss Electric Ltd.).

Intrinsic milk enzymes were inactivated (denatured) by heating the milk samples in a water bath. Isocitrate in milk is found in a free state and in an esterified state. Samples analyzed for free isocitrate were heat-treated at 75°C for 10 min. Samples analyzed for total isocitrate were adjusted to pH 10 with 1 mol/L of NaOH and heat-treated at 96°C for 20 min, because heat and the alkaline environment breaks the chemical ester bond between lactate and other intrinsic components. Thus, total isocitrate is the sum of free isocitrate and esterified isocitrate.

Isocitrate Analysis

Two reagents were necessary for the detection of isocitrate by fluorometry. Reagent 1 contained the enzyme that mediates the conversion of isocitrate to 2-oxoglutarate (i.e., ICDH; EC 1.1.1.42), Tris-buffer, pH 7.2, NADP⁺, and Mn-ions that activates the enzyme. Reagent 2 contained the necessary fluorophore, resazurin,

and the enzyme mediating the reduction of resazurin, diaphorase (EC 1.6.99.1), both dissolved in Tris-buffer with Triton X-100 (Merck, Darmstadt, Germany).

Eighty microliters of diluted sample (1:2 water) was pipetted into a microplate, then 60 μ L of reagent 1 was added ($t = 0$) and the mixture was incubated 3 min (incubation 1) at room temperature. After this, 40 μ L of reagent 2 was added and the plate was incubated for an additional 6 min (incubation 2) and read (excitation: 544 nm; emission: 590 nm) in a fluorometer (FluoStar Galaxy, BMG Labtechnology, Germany). Incubation conditions for incubation 1 were: sample fraction = 0.19; isocitrate maximum = 0.076 mmol/L; Mn⁺⁺ = 0.77 mmol/L; Tris-buffer (pH 7.2) = 31 mmol/L; ICDH activity = 0.11 U/mL; β -NADP⁺ = 0.123 mmol/L. Incubation 2 conditions were: diaphorase activity = 2.2 U/L; resazurin = 0.28 mmol/L; Triton X-100 = 0.0024%; Tris-buffer = 34 mmol/L.

Analytical Details

The ICDH oxidizes isocitrate to 2-oxo-glutarate, whereas NADP⁺ is reduced equivalently to NADPH + H⁺ at pH 7.2. The reaction is activated by Mn ions. The developed NADPH + H⁺ equivalently reduced resazurin to resorufin, mediated by the enzyme diaphorase; the amount of resorufin is detected by fluorometry and compared with a standard curve. All dilutions and additions of reactants were performed in a laboratory robotic system (Biomek 2000; Beckman Coulter, Pasadena, CA).

Standards and Controls

Standards were prepared in isocitrate-free milk: 45 mL of milk were supplied with 5 mL of 1 mol/L Tris-buffer, pH 7.2, 25 mg of Mn-sulfate, 14 U of ICDH, and 10 mg of NADP⁺ and incubated for 60 min at 40°C. Terminally, 1.0 mL of hydrogen peroxide (15%) was added, mixed immediately, and the mixture was heat-treated at 75°C for 10 min. Standards were prepared by addition of DL-isocitrate (Acros 205010010, MW 258.07; Acros Organics, Geel, Belgium) to the isocitrate-free milk; concentrations of standards were 0, 0.02, 0.04, 0.08, 0.10 0.20, 0.30, and 0.40 mmol/L of D-isocitrate. Control samples were produced likewise, using 0.069 and 0.227 mmol/L of D-isocitrate. Standards and controls were measured in duplicate on every microplate.

Verification of the Assay

Four hundred thirty milk samples were analyzed for both total isocitrate and free isocitrate according to

Figure 1. Four hundred thirty random milk samples were analyzed for total isocitrate (abscissa) and free isocitrate (ordinate) according to the procedures described herein. Color version available in the online PDF.

the scheduled procedure to elucidate the circumstances about the ratio of the 2 forms. The present fluorometric analyses of isocitrate were validated by means of several initiatives comprising reference measurements by fluorometry and spectrophotometry on precipitated milk samples, investigation of the linearity of the method, and checking the accuracy and the precision.

Reference measurements by fluorometry were completed by using 40 milk samples in analysis for free and total isocitrate by the presented fluorometric assay (3 replicates). Concurrently, parallel milk samples were precipitated with 3% metaphosphate or 12% perchloric acid (0.5 mL of acid to 2.0 mL of milk), centrifuged at room temperature, $2,000 \times g$, for 10 min, and the supernatant subsequently neutralized with 2 mol/L of potassium carbonate. The neutralized (clear) supernatants were analyzed by fluorometry compared with standards treated likewise (metaphosphate- and perchloric acid-treated, respectively). Furthermore, samples precipitated by perchloric acid were analyzed for isocitrate by spectrophotometry (Advia 1650 Autoanalyzer, Siemens Diagnostics; Siemens Medical Solutions, Tarrytown, NY). The analytical conditions were: sample fraction = 0.083; $Mn^{++} = 0.62$ mmol/L;

Tris-buffer (pH 7.2) = 62 mmol/L; ICDH = 0.22 U/mL; β -NADP⁺ = 0.148 mmol/L.

Linearity of the fluorometric assay was checked by spiking milk samples with pure isocitrate. One hundred forty-four samples were spiked with 0.08, 0.09, 0.10, 0.16, 0.18, or 0.20 mmol/L of isocitrate, respectively. Linearity of the assay was also investigated by merging samples of known isocitrate content; 1 low sample was mixed with 1 high sample, then (1) 48 samples were analyzed for total isocitrate and (2) 96 samples were tested for free isocitrate. The expected isocitrate concentration was subsequently compared with the actual measurement.

Intra-assay validation of free isocitrate was conducted on 288 random samples replicated 3 times on the same microplate. Interassay validation and accuracy was assayed on 17 microplates, where low (0.069 mmol/L) and high control (0.227 mmol/L) were measured repeatedly. The potential interference of citrate on the present analysis of isocitrate was investigated for both free and total isocitrate, where 16 ordinary, fresh milk samples were spiked with 0, 10, or 20 mmol/L of citrate (pH 7.2; 3 replicates) and analyzed according to the described procedure. Pearson correlations were used to

Table 1. Forty milk samples were analyzed for total and free isocitrate using both the fluorometric and the spectrophotometric methods¹

Item	Fluorometry			Spectrophotometry	
	Whole milk		Precipitated milk	Precipitated milk	
	Total isocitrate	Free isocitrate		Metaphosphate	Perchloric acid
Mean, ² mmol/L (range)	0.184 (0.109–0.343)	0.162 (0.094–0.283)		0.148 (0.099–0.259)	0.205 (0.135–0.374)
Correlation between total isocitrate in whole milk (x) and precipitated milk (y)				$y = 0.70x + 0.02$ ($r = 0.97$)*	$y = 0.88x + 0.04$ ($r = 0.97$)*
Correlation between free isocitrate in whole milk (x) and precipitated milk (y)				$y = 0.78x + 0.02$ ($r = 0.93$)*	$y = 0.99x + 0.05$ ($r = 0.94$)*

¹Samples and standards were precipitated for fat and protein by 3% metaphosphate or 12% perchloric acid and measured for isocitrate by fluorometry (both metaphosphate and perchloric acid precipitated samples) or by spectrophotometry (perchloric acid precipitated samples).

²n = 40.

* $P < 0.001$.

determine the correlation among free and total isocitrate in milk with DIM, milk yield, time since last milking, and components in milk (i.e., fat, protein, lactose, citrate, and SCC). The effect of breed was determined by 2-sided *t*-test.

RESULTS

Total isocitrate (i.e., after liberation of the esterified acid) ranged between 0.093 and 0.378 mmol/L for the analyzed material (mean 0.179 mmol/L; $n = 430$). Correspondingly, free isocitrate ranged between 0.072 and 0.333 mmol/L (mean 0.147). The ratio between free and total isocitrate was between 0.729 and 0.939 ($P_{2-P_{98}}$ interpercentile, where P = percentile; mean 0.816). The results are summarized in Figure 1.

Reference measurements by fluorometry and spectrophotometry are shown in Table 1. Fluorometric determination of milk samples precipitated by metaphosphate gave numerically lower values than direct fluorometric determinations of both free isocitrate and total isocitrate. However, whole and precipitated milk were significantly correlated ($P < 0.001$) for both free and total isocitrate. Milk samples precipitated by perchloric acid showed levels of isocitrate comparable to the direct measurements of total isocitrate ($P < 0.001$), both measured by fluorometry and spectrophotometry. Fluorometry of precipitated samples gave on average ~9% lower values than spectrophotometry. Fluorometry on whole milk samples were ~11% lower than spectrophotometry on precipitated samples; see Table 1 for details.

The linearity check of the fluorometric isocitrate assays is given in Table 2. Measurement of samples spiked with known concentrations of pure isocitrate gave results similar to the expected. Results from measurements of free isocitrate in merged samples (low + high, 1:1) revealed on average 9% higher values than expected (0.191 vs. 0.175 mmol/L), whereas total isocitrate measurements gave on average 3% higher values than expected.

The intra-assay precision varied from 0.12 to 7.0 (CV, $n = 288$), mean CV was 1.8. The interassay precision was for low-control samples 8.6 (CV, $n = 17$), and for high-control samples 5.8 (CV, $n = 17$). The inaccuracy was 0.0004 and 0.0020 mmol/L, respectively, for low (0.069 mmol/L) and high (0.227 mmol/L) controls, resulting in a relative bias of 0.6 and 0.9%, respectively.

Samples spiked with 0 mmol/L of citrate ranged from 0.127 to 0.214 mmol/L of total isocitrate (mean 0.167 mmol/L). Samples spiked with 10 mmol/L of citrate were on average 2.8% higher ($r = 0.987$; $P < 0.001$) and samples spiked with 20 mmol/L of citrate were on average 0.3% lower in total isocitrate ($r = 0.938$;

Table 2. Linearity of the fluorometric isocitrate assay was investigated by spiking of milk samples with known concentrations of isocitrate (A; 0.08; 0.09; 0.010; 0.16; 0.18; or 0.20 mmol/L) or by merging (1:1) milk samples low in isocitrate with milk samples high in isocitrate, and then analyzed for free isocitrate (B) or total isocitrate (C)

Item	Analysis	n	Isocitrate mmol/L, mean (range)		Equation
			Measured, y	Expected, x	
A	Spiking of milk samples with isocitrate	144	0.274 (0.186–0.444)	0.269 (0.188–0.471)	$y = 0.98x + 0.000^*$
B	Free isocitrate	48	0.191 (0.107–0.303)	0.175 (0.085–0.273)	$y = 0.89x + 0.036^*$
C	Total isocitrate	24	0.182 (0.109–0.276)	0.177 (0.103–0.286)	$y = 0.96x + 0.012^*$

* $P < 0.001$.

$P < 0.001$). Samples spiked with 0 mmol/L of citrate ranged from 0.102 to 0.166 mmol/L of free isocitrate (mean 0.133 mmol/L). Samples spiked with 10 mmol/L of citrate were on average 1.8% higher ($r = 0.961$; $P < 0.001$) and samples spiked with 20 mmol/L of citrate were on average 2.3% lower in free isocitrate ($r = 0.982$; $P < 0.001$). Coefficient of variation was in all instances below 6% for the replicates.

The comparisons between isocitrate and basic milking data and measured standard variables are shown in Table 3. The mean isocitrate content of the samples was 0.168 mmol/L (P_2 – P_{98} interpercentile = 0.070–0.293). Milk from Danish Holstein cows (984) contained significantly less isocitrate than milk from Danish Jersey cows (760; i.e., 0.134 vs. 0.211 mmol/L; $P < 0.001$). The isocitrate concentration is positively correlated to the fat, protein, and citrate content in milk (Table 3).

DISCUSSION

Milk may contain indigenous enzymes able to use NADP⁺ as co-factor, thereby producing NADPH + H⁺ and, second, the fluorophore we are detecting in

the present setup. Such a parallel production would obviously obstruct the present detection of isocitrate. However, few relevant enzymes are mentioned in the literature (e.g., Farkye, 2003). Malic enzyme (EC 1.1.1.40), 6-phosphoglucuronate dehydrogenase (decarboxylating; EC 1.1.1.44), and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) are listed, and also mentioned by Bauman et al. (1970), Gumaa et al. (1973), and Grigor and Hartman (1985) in relevant studies. However, the activity of these enzymes is measured in mammary gland cytosol, from cow or sheep or in sow or rat milk. Furthermore, the essence of the 2 first articles is explicit that ICDH-activity in the mammary gland cytosol is more than 10 times higher than the other enzymes, especially for ruminants. Therefore, we chose to heat-treat the milk samples (75°C for 10 min) to denature all indigenous enzymes (including ICDH) to avoid both unwanted production and oxidation of already-produced NADPH, because both circumstances would interfere with the assessment of isocitrate.

Approximately 20% of the total isocitrate in milk seems to be esterified to other milk components or even lactonized. We are unsure if the esterified fraction, be-

Table 3. Milk samples were analyzed for standard variables and free isocitrate and correlated (Pearson correlation, r) mutually and to basic milking data¹

Item	DIM	Yield	Time	Fat	Protein	Lactose	Citrate	log SCC
Isocitrate	0.120	−0.359	0.172	0.632	0.655	−0.217	0.553	0.067
n	1,725	1,725	1,485	1,706	1,708	1,708	782	1,710
DIM		−0.195	0.093	0.303	0.349	−0.113	0.321	0.037
n		1,725	1,725	1,706	1,708	1,708	782	1,710
Yield			0.527	−0.509	−0.532	−0.008	−0.297	−0.137
n			1,485	1,706	1,708	1,708	782	1,710
Time				0.004	0.125	−0.001	−0.025	−0.086
n				1,485	1,485	1,485	782	1,485
Fat					0.764	−0.221	0.527	0.094
n					1,706	1,706	782	1,706
Protein						−0.257	0.473	0.103
n						1,708	782	1,708
Lactose							−0.307	−0.247
n							782	1,708
Citrate								0.031
n								782

¹Isocitrate = mmol/L of milk; yield = milk yield in kilograms for the actual milking; time = time since last milking (h); fat, protein, lactose, citrate = % in milk.

tween 27 and 6% (P_2 – P_{98} inter percentile, $n = 430$) obtained in our study, could be linked to nutritional or physiological conditions in the cow. Literature on this subject is scarce and future experiments and studies will have to elucidate this question.

Addition of citrate to the reaction medium—comparable to the natural content—did not influence the results of either free or total isocitrate. Citrate is not a substrate for the ICDH enzyme supplied to the assay, and obviously no transformation of citrate to isocitrate occurred in the sample. If milk contains aconitase [i.e., the catalytic step between citrate and isocitrate, as postulated by Faulkner et al. (1986)], then the aconitase activity is most likely destroyed during the initial heat treatment.

The obtained results concerning linearity test, precision, and accuracy are acceptable for analytic as well as descriptive use. Both fluorometric as well as spectrophotometric reference analyses based on perchloric acid precipitations of protein and fat showed results comparable to the present direct measurement of total isocitrate.

All studies and publications until now have used the spectrophotometric analyses on perchloric acid-precipitated samples (e.g., Faulkner, 1980; Chaiyabutr et al., 1981; Hurtaud et al., 1998, 2000; Rigout et al., 2002). These former studies report isocitrate levels comparable to the present study although the number of animals and samples involved in these studies are much more limited. The traditional procedure based on precipitation and centrifugation is time and material consuming and may thereby restrict the number of analyses. The present direct fluorometric assay is considerably easier to perform. This opens for a markedly higher analytical capacity per day including in-line measurements directly from the herd.

Numerous studies connect variation in isocitrate in dairy milk to FA synthesis during lactation. Garnsworthy et al. (2006) concluded that the variation in milk citrate with stage of lactation is related to de novo synthesis of FA and that the relationship is independent of diet and milk yield. The present study shows that milk citrate and isocitrate are directly proportional to DIM ($P < 0.001$; see Table 3), but both inversely proportional to milk yield ($P < 0.001$). The regulation and expression of ICDH in the mammary gland has been studied (Seery and Farrell, 1990; Farrell et al. 1990), as well as in relation to gestation and lactation (Farrell et al., 1987). The NADP⁺-dependent form (ICDH1) is elevated postpartum where milk FA synthesis starts. In addition, Liu et al. (2006) demonstrated that ICDH1 mRNA levels in bovine mammary tissue increased by 2.3-fold after parturition compared with late pregnancy. Furthermore, studies strongly suggest that long-chain

acyl-CoA inhibit the NADPH-producing ICDH enzyme, whereas short-chain acyl-CoA do not (Farrell et al. 1995). This indicates that the ICDH enzyme activity is depressed when dietary FA are available from the blood, and the depression is abolished or reduced when short-chain FA are produced in the mammary gland by the de novo synthesis.

Basic biochemistry, former studies, and the present study strongly indicate that the presence of isocitrate in milk may reflect the energy situation in the udder. Further studies may elucidate if isocitrate in milk has a potential as indicator of energy status in the dairy cow and elaborate more on the physiological background for its variation in milk.

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