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Technical note: Rapid method for determination of amino acids in milk

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ABSTRACT

A rapid method for measurement of amino acids in milk was developed and validated. The method included a first step of milk protein hydrolysis, followed by the derivatization and separation of amino acids by HPLC. Six combinations of hydrolysis agent and temperature-time conditions were compared with a reference method; derivatization procedures as well as HPLC separation were improved. Hydrolysis of milk samples with 6 *N* HCl at 160°C for 60 min resulted in no significantly differences compared with the reference method but allowed the analysis of a greater number of milk samples in a short time. In addition, this method was characterized by high precision, low repeatability uncertainty, and high accuracy for all amino acids evaluated; the recovery mean value of the single amino acids was 98.38%. The proposed method is, therefore, accurate, simple, rapid, and suitable for large numbers of milk samples.
Key words: amino acid, HPLC, derivatization, milk

Determination of the amino acid profile in milk is essential for qualitative evaluation of peptides and proteins that can affect the chemical and nutritional properties of milk. The measurement of amino acid composition is a complex analytical process, consisting of 2 steps: 1) hydrolysis of substrate; and 2) derivatization and chromatographic analyses. As reported by Fountoulakis and Lahm (1998), hydrolysis is a critical step for this analysis, being the main source of analytical errors. The official reference procedure is a liquid-phase hydrolysis performed in constantly boiling 6 *N* HCl under vacuum at 110°C for 24 h (AOAC, 1994). The long time required for hydrolysis could be a limitation of the official method. Amino acids are derivatized before analysis, which could also represent a critical step of the protocol. In particular, the choice of an optimal combination of sample, buffer, and fluorescence agents is necessary to obtain an adequate final pH value to improve the derivatization efficiency. Although a reference method is reported for amino acid analysis in food products, no studies have been carried out on the determination of AA in milk as a specific food substrate. The objective of this technical note is to provide a method for the determination of AA in milk. For this purpose, 6 combinations of hydrolysis agent and analysis conditions were compared with the reference procedure. For all proposed methods, the derivatization reaction and HPLC chromatographic separations was performed according to Henderson et al. (2000), with modification.

Individual milk samples (at least 200 mL) were collected from 12 cows; total protein content was determined using an infrared spectrophotometer (MilkoScan 133B, Foss Electric, Hillerød, Denmark; IDF, 1990) and was 3.35% ± 0.35 SEM. A pretrial was conducted combining hydrolysis agent [HCl and methanesulfonic acid (**MSA**)], time of analysis (from 24 h to 45 min), and temperature (from 110 to 160°C). All analytical methods were compared with the reference hydrolysis method (6 *N* HCl at 110°C for 24 h), and 6 methods were chosen on the basis of a total AA recovery >70% compared with the reference method. The 6 selected methods are showed in Table 1. Each hydrolysis method was repeated 3 times for each of the 12 cow milk samples. The hydrolysis of milk proteins was performed in Pyrex microcapillary tubes (Pierce Chemical Company, Rockford, IL) under vacuum and heated at the temperatures reported in Table 1 using a conventional oven. The same volumes of milk (250 μL) and hydrolysis agent (250 μL) were used for each method,. After hydrolysis, the tubes were cooled and the samples were filtered using Spartan-HPLC 13-mm syringe filters (0.45 μm, 30 mm; Schleicher and Schuell, Dassel, Germany); the filtrate was diluted with distilled water (1:20 vol/vol) in amber glass vials.

Amino acids were determined by HPLC. Before injection, AA were derivatized on-line using *o*-phthaldehyde (**OPA**) for primary AA and 9-fluorenylmethyl chloroformate (**FMOC**) for secondary AA according to the method of Henderson et al. (2000), modified to optimize the parameters for milk analysis. The following

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Table 1. Combinations of hydrolysis agent and conditions (time and temperature) tested

Method	Hydrolysis agent	Hydrolysis conditions	
		Temperature (°C)	Time
Ref.[1]	Hydrochloric acid 6 *N*	110	24 h
A	Methanesulfonic acid 4 *N*	110	24 h
B	Methanesulfonic acid 4 *N*	150	90 min
C	Hydrochloric acid 6 *N*	160	120 min
D	Hydrochloric acid 6 *N*	160	105 min
E	Methanesulfonic acid 4 *N*	160	45 min
F	Hydrochloric acid 6 *N*	160	60 min

[1]Ref = reference method (AOAC, 1994).

volumes were injected: 26 μL of borate buffer mixed with 0.1 μL of sample, 0.3 μL of OPA, 0.3 μL of FMOC reagent, and 16 μL of HPLC-grade water. The HPLC system (1100 series, Agilent Technologies, Waldbronn, Germany) was composed of a binary pump equipped with micro vacuum degasser, thermostat-controlled autosampler, column compartment, a fluorescence detector (model G1321A), and a diode array detector (model G1315A). The analyses were performed using a Zorbax Eclipse AAA column (150 × 4.6 mm i.d., prepacked with 3.5 μm particles; Palo Alto, CA); the column temperature was set at 40°C. The mobile phase comprised a 40 m*M* $NaH_2PO_4 \cdot H_2O$ solution (phase A) and a mixture of water, methanol, and acetonitrile (10:45:45 vol/vol/vol; phase B). The elution of samples was performed at a flow rate of 2.0 mL/min by gradient elution, and the total run time was 30 min. Fluorescence detection was carried out at 340 (excitation) and 450 nm (emission). The UV diode array detector was used to determine cystine at 338 nm. Individual amino acid peaks were identified by comparing their retention times with those of standards (Sigma-Aldrich, St. Louis, MO). Nine hundred microliters of standard mix solution of 17 AA (Asp, Glu, Ser, His, Gly, Thr, Arg, Ala, Tyr, Cys, Val, Met, Phe, Ile, Leu, Lys, and Pro) at a concentration of 1 nmol/μL and 100 μL of Asn, Gln, and Trp at 9 nmol/μL were mixed in a vial to obtain standard solutions with a concentration of 900 pmol/μL for each amino acid.

Results are reported as milligrams of amino acid per gram of total AA. For each single amino acid, precision (CV %), recovery, repeatability uncertainty, linearity, limit of determination (**LOD**), and limit of quantification (**LOQ**) were evaluated. Total recovered AA were determined as the sum of each amino acid on the protein content, as reported by Davis et al. (1994). Recovery percentage of each amino acid was evaluated by adding 1 g of lysozyme lyophilized powder (Sigma-Aldrich) to an aliquot of milk sample. The amount of AA detected in the sample with added lysozyme minus the amount of AA detected in a sample without lysozyme gave the percentage recovery of each amino acid (Tristam, 1953). Repeatability uncertainty was studied on 6 analyses of the same sample. The standard solution was diluted with 0.1 *N* HCl to obtain the following concentrations: 450, 225, and 112.5 pmol/μL; linearity of the analysis was determined from 112.5 to 900 pmol/μL of standard solution by plotting peak response (area of the amino acid peak divided by the area of the internal standard peak) against concentration. The LOD and LOQ were calculated according to the following equations (Miller and Miller, 1993):

$$\text{LOD} = 3.3\ s_a/b$$

$$\text{LOQ} = 10\ s_a/b,$$

where s_a is the standard deviation of the intercept and b is the slope of the regression line, obtained from the calibration curve. The repeatability uncertainty was calculated according to Hund et al. (2001).

The effect of the proposed methods and the reference method on amino acid levels and recovery percentages was analyzed using the GLM procedure of the SAS software package (SAS Institute Inc., Cary, NC). When significant effects were found (at $P < 0.05$ unless otherwise noted), Student's *t*-test was used to locate significant differences between means.

Table 2 shows the AA content of cow milk samples determined using the 6 different hydrolysis methods and the reference method. The concentration of each amino acid was significantly affected by hydrolysis method. Increases of temperature and reduction of hydrolysis times gave variable results. Although similar hydrolysis conditions were used in methods E and F, the hydrolysis agent (MSA vs. HCl) used was a significant factor in determination of milk AA. Indeed, milk amino acid concentrations determined by method F (using HCl) were within the range reported for bovine milk by other authors (Davis et al., 1994). On the contrary, results obtained with method E (using MSA) were not similar to reported values in the literature. In addition, be-

Table 2. Mean value of AA of cows' milk (mg/g of protein) determined by HPLC using different hydrolysis methods

	Method[1]							
Amino acid	Ref.	A	B	C	D	E	F	SEM
Alanine	40.47^{a}	41.90^{a}	40.85^{a}	40.45^{a}	40.06^{a}	34.87^{b}	32.62^{c}	0.62
Arginine	42.91^{b}	45.12^{a}	41.77^{bc}	41.13^{c}	40.84^{c}	38.48^{d}	36.29^{f}	0.58
Aspartic acid	75.44^{d}	84.28^{b}	83.27^{b}	82.15^{c}	92.04^{a}	75.54^{d}	73.84^{d}	0.65
Cysteine	7.53^{a}	3.14^{e}	5.44^{c}	4.85^{d}	5.21^{c}	6.30^{b}	7.66^{a}	0.15
Glutamic acid	209.03^{e}	269.63^{a}	216.58^{d}	240.28^{c}	254.86^{b}	198.929^{f}	211.68^{e}	1.20
Glycine	26.39^{c}	22.30^{d}	28.09^{b}	29.88^{a}	20.148^{e}	18.84^{e}	19.90^{e}	0.48
Histidine	37.90^{a}	27.55^{e}	33.08^{c}	24.56^{f}	28.92^{d}	35.43^{b}	26.53^{e}	0.45
Isoleucine	51.65^{a}	47.12^{b}	47.86^{b}	39.70^{c}	37.96^{c}	46.74^{b}	54.12^{a}	1.18
Leucine	98.70^{c}	109.53^{a}	104.16^{b}	95.33^{d}	95.61^{d}	95.02^{d}	98.20^{c}	0.82
Lysine	86.71^{a}	66.31^{c}	56.32^{e}	85.58^{a}	63.51^{d}	76.65^{b}	85.25^{a}	0.65
Methionine	24.67^{e}	32.28^{ab}	31.02^{b}	29.27^{c}	26.20^{d}	33.34^{a}	25.40^{de}	0.58
Phenylalanine	45.75^{d}	48.98^{c}	47.44^{cd}	42.23^{e}	53.80^{a}	51.34^{b}	46.31^{d}	0.75
Proline	45.63^{f}	21.98^{g}	72.22^{c}	48.34^{e}	51.98^{d}	96.91^{a}	93.12^{b}	0.71
Serine	61.34^{a}	60.31^{b}	62.88^{a}	60.90^{b}	51.78^{c}	49.36^{d}	53.12^{c}	0.65
Threonine	54.94^{a}	53.83^{a}	44.82^{d}	50.81^{b}	46.04^{c}	40.89^{e}	44.81^{d}	0.44
Tryptophan	13.52^{a}	12.35^{b}	11.45^{b}	12.05^{b}	11.52^{b}	12.54^{b}	14.11^{a}	0.4
Tyrosine	48.42^{b}	49.81^{a}	44.47^{d}	44.94^{d}	47.90^{b}	46.47^{c}	47.58^{b}	0.42
Valine	48.53^{b}	15.90^{f}	42.68^{d}	39.56^{e}	46.08^{c}	55.83^{a}	50.61^{b}	0.71
Total AA (mg/100 mL)	2,917^{b}	2,515^{g}	2,623^{e}	2,800^{c}	2,575^{f}	2,689^{d}	3,200^{a}	18.00
Recovery (%)	87.09^{b}	75.08^{d}	78.32^{d}	83.61^{bc}	76.89^{d}	80.29^{cd}	95.52^{a}	1.55

$^{a–g}$Means within a row with different superscripts differ ($P < 0.05$).

[1]Ref. = reference method (AOAC, 1994); conditions for other methods are shown in Table 1.

cause MSA is not volatile, it cannot be evaporated after hydrolysis; hence, greater sample amounts have to be used for hydrolysis. Conversely, HCl can be evaporated after hydrolysis, so the hydrolysate is recovered in a small volume (Weiss et al., 1998). To test the accuracy of the proposed methods, analytical recovery was evaluated. As can be observed in Table 2, recovery of total milk AA was 95.52% for method F compared with 87.10% for the reference method; the other methods showed lower recovery percentages ranging from 75.08 to 83.61%. Table 3 shows recovery percentages of each amino acid for the tested hydrolysis methods. Method F was characterized by higher accuracy than the reference method for alanine ($P < 0.01$), arginine, glycine, histidine, proline, serine, and threonine ($P < 0.001$), whereas no significant differences were found for the

Table 3. Mean value of recovery percentages of different hydrolysis methods

	Method[1]							
Amino acid	Ref.	A	B	C	D	E	F	SEM
Alanine	89.05^{c}	88.90^{c}	88.07^{c}	89.25^{c}	89.40^{c}	92.70^{b}	95.20^{a}	0.25
Arginine	96.51^{b}	90.50^{c}	75.90^{f}	67.50^{g}	86.10^{d}	84.70^{e}	98.80^{a}	0.30
Aspartic acid	95.52^{a}	86.10^{b}	85.15^{b}	78.30^{d}	81.58^{c}	95.65^{a}	96.40^{a}	0.38
Cysteine	99.05^{a}	84.67^{f}	86.78^{e}	88.55^{d}	89.67^{c}	92.34^{b}	98.99^{a}	0.18
Glutamic acid	99.85^{a}	89.80^{c}	91.30^{b}	81.30^{e}	85.70^{d}	78.05^{f}	98.99^{a}	0.35
Glycine	90.45^{d}	92.55^{c}	71.70^{e}	72.10^{e}	95.10^{b}	94.50^{b}	97.20^{a}	0.28
Histidine	90.55^{c}	96.50^{a}	91.25^{c}	95.12^{b}	94.65^{b}	91.15^{c}	97.30^{a}	0.36
Isoleucine	98.85^{a}	97.05^{b}	96.88^{b}	91.55^{c}	90.67^{c}	97.35^{b}	99.05^{a}	0.22
Leucine	96.50^{a}	85.55^{e}	88.55^{d}	90.25^{c}	91.15^{b}	90.55^{bc}	96.98^{a}	0.26
Lysine	98.05^{a}	92.55^{b}	91.05^{c}	96.95^{a}	88.50^{d}	86.47^{e}	97.65^{a}	0.44
Methionine	99.15^{a}	89.88^{d}	90.75^{c}	92.05^{b}	98.85^{a}	91.25^{bc}	99.55^{a}	0.27
Phenylalanine	97.92^{a}	87.55^{c}	96.55^{b}	85.24^{d}	85.95^{d}	85.45^{d}	98.15^{a}	0.22
Proline	85.25^{d}	75.25^{g}	88.85^{c}	80.67^{e}	78.61^{f}	95.45^{b}	99.05^{a}	0.18
Serine	96.12^{b}	91.55^{c}	86.45^{e}	90.85^{c}	96.55^{b}	88.47^{d}	98.55^{a}	0.24
Threonine	95.65^{b}	90.63^{c}	97.72^{a}	89.05^{d}	88.56^{d}	85.24^{e}	98.57^{a}	0.31
Tryptophan	98.55^{a}	95.35^{b}	95.20^{b}	94.85^{b}	93.05^{c}	92.55^{c}	99.02^{a}	0.25
Tyrosine	98.84^{a}	90.25^{c}	81.05^{f}	82.35^{e}	94.55^{b}	85.45^{d}	99.10^{a}	0.15
Valine	97.85^{a}	79.56^{e}	82.55^{d}	78.55^{f}	84.52^{c}	90.55^{bc}	98.17^{a}	0.21

$^{a–g}$Means within a row with different superscripts differ ($P < 0.05$).

[1]Ref = reference method (AOAC, 1994); conditions for other methods are shown in Table 1.

Table 4. Linearity parameters, limits of detection and quantification (LOD and LOQ), and performance parameters of the analytical method proposed (method F)

Amino acid	r^2	LOD	LOQ	CV (%)	Repeatability uncertainty (%)
Alanine	0.998	0.75	2.28	3.8	1.5
Arginine	0.998	1.37	4.15	1.7	0.7
Aspartic acid	0.996	1.27	3.87	1.9	0.8
Cysteine	0.996	1.78	5.4	4.6	1.8
Glutamic acid	0.996	1.53	4.63	0.7	2.8
Glycine	0.997	0.97	2.95	1.4	0.6
Histidine	0.997	1.45	4.39	1.1	0.5
Isoleucine	0.997	1.21	3.66	2.3	0.9
Leucine	0.997	1.2	3.62	0.6	0.2
Lysine	0.99	2.59	7.71	4.3	1.7
Methionine	0.997	1.4	4.25	2.9	1.2
Phenylalanine	0.998	1.3	3.93	1.6	0.6
Proline	0.991	1.74	5.26	4	1.6
Serine	0.998	0.76	2.31	0.8	0.3
Threonine	0.997	1.1	3.32	1.7	0.6
Tryptophan	0.998	1.52	3.59	2.5	1
Tyrosine	0.998	1.6	4.86	0.9	0.3
Valine	0.998	0.91	2.76	0.7	0.3

other AA. It is worth noting that the mean recovery value of the single AA detected with method F was 98.38%, ranging between 97.20 and 99.84%, except for alanine (95.20%).The linearity parameters expressed as determination coefficients and the relative LOD and LOQ of each amino acid detected after hydrolysis with method F are reported in Table 4. All the AA showed good linearity with coefficients of determination (r^2) >0.99. The highest LOD and LOQ were recorded for lysine, whereas the lowest values were recorded for alanine and serine. The coefficients of variation related to the AA concentrations registered were <4.6%, with a mean value of 2.1%; this could be considered an index of high repeatability, according to the European Commission (2002).

To summarize, hydrolysis of milk samples using 6 *N* HCl at 160°C for 60 min (method F) permits good quantification of milk AA associated with increased accuracy together with the possibility to analyze an increased number of milk samples in short time. The proposed modification for derivatization procedure allowed us to obtain good chromatographic resolution and excellent asymmetry of the peaks. Therefore, method F was characterized by high precision and low repeatability uncertainty for all AA evaluated.

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