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Optimization of the analytical extraction of polyamines from milk

Juliana Cristina Sampaio Rigueira[a], Maria Isabel Rodrigues[b], Maria Beatriz Abreu Gloria[a,*]

[a] *LBqA – Laboratório de Bioquímica de Alimentos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Belo Horizonte, MG, 31270-901, Brazil*
[b] *Departamento de Engenharia de Alimentos, Faculdade de Engenharia de Alimentos, UNICAMP, Campinas, SP, 13081-970, Brazil*

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ABSTRACT

Polyamines play an important role as growth promoters, in the maturation of the intestinal tract of infants and in the modulation of the immune response; consequently, the importance of polyamines in the diet of infants and adults is well established. However, information on the occurrence and levels of polyamines in cow's milk and milk from other species (including human milk) is contradictory. Furthermore, the methods used for the extraction of amines from these samples vary widely. Therefore, a method for the accurate analysis of amines in milk from different species and in milk products is needed. A sequential strategy of experimental designs was used to optimize the analytical extraction of polyamines from milk. The dependent variables that significantly affected the recoveries were screened through a Plackett–Burman design. Sulfosalicylic acid (SSA) provided better recoveries compared to trichloroacetic acid. Centrifugation time and speed during extraction were independent variables. The Central Composite Rotational Design used to optimize the dependent variables indicated that the optimal conditions for the extraction of polyamines were 40 s vortexing, four successive extractions, and an SSA concentration of 1.5%. These conditions provided recoveries ≥92.8% and CV ≤ 5.8%. The experiments confirmed the predicted results, indicating that the optimized conditions and models used were effective in the determination of amines from milk. Samples of raw milk and milk beverages were observed to be poor sources of spermine and spermidine, whereas human milk contained both amines.

1. Introduction

Polyamines are aliphatic organic bases that belong to a larger group of amines called bioactive or biologically active amines. The most important ones are spermidine and spermine, which play important roles in metabolic and physiologic functions in living organisms [1,2]. Intracellular polyamines levels are essential for many cell functions, such as protein, RNA and DNA synthesis, and stabilization of DNA [1,3–5]. Furthermore, higher polyamines levels are correlated with increased antioxidant activity [6,7]

Polyamines are formed from the decarboxylation of ornithine and arginine by ornithine decarboxylase (ODC) and arginine decarboxylase, respectively. They can also be formed from citrulline; however, independent of the source, the diamine putrescine is a necessary intermediate [8,9].

Polyamines are naturally present at low concentrations in living organisms as well as in foods [3]. However, the amount synthesized *in situ* may not be enough to meet the requirements. Studies have indicated that extracellular sources of polyamines are also important in metabolic processes [2,10].

The importance of polyamines in cellular growth, differentiation and proliferation is well established in humans. There is a high rate of polyamines synthesis in fetuses and during the first days of an infant's life, which is associated with their role in cell growth and in the development of the digestive system [7,11]. The synthesis of polyamines, especially spermidine, gradually increases in the mammary gland during pregnancy and lactation [4]. In milk, the polyamines function as growth factors in the lumen, promote intestinal maturation, reduce mucosal permeability to macromolecules and prevent food allergies [12–14].

Because polyamines are taken up by tissues undergoing rapid growth rates, they are also taken up by tumor cells. Therefore, a polyamine deprivation regimen, in combination with inhibitors of ODC and polyamine oxidase, has been shown to reduce the growth of tumors [15].

The information regarding amines in cow's milk is contradictory. Gloria et al. [16] found a prevalence of spermine, whereas Motyl et al. [17] reported a prevalence of spermidine in raw milk. It is important to determine the levels of polyamines in milk and dairy products to be able to accurately recommend diets for individuals with specific needs. The most common techniques for the determination of amines involve extraction, separation and quantification. Extraction consists of the removal of amines from the matrix and is considered a critical step [18–20]. Several methods for extraction of amines from milk have been described in the literature [16,17,21].

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* Corresponding author. Tel.: +55 31 34096926; fax: +55 31 34096911.
E-mail address: mbeatriz@ufmg.br (M.B.A. Gloria).

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Several steps are taken including addition of solvents, agitation, centrifugation, partition and filtration. However, there is still no consensus on which are the best solvent and conditions for extraction. Reliable methods are needed to accurately determine these compounds in milk and products.

Plackett–Burman (PB) designs are useful to screen the main factors from a large number of variables that can affect the response. Therefore, they can be used in preliminary studies to select variables that can be fixed or eliminated in further optimization processes. In addition, response surface methodology (RSM) is an efficient experimental tool by which optimal conditions may be determined in a multivariable system [22,23].

The objective of this work was to optimize the extraction procedure for the analytical determination of polyamines in milks and dairy products. A PB design was useful in screening main factors from a large number of variables that could affect the extraction of polyamines. The RSM of the selected factors was used as a tool to obtain the best extraction conditions. The optimized method was validated and used in the analysis of milks and dairy beverages. The method was reliable and also provided reduced analysis time and cost.

2. Materials and methods

2.1. Samples and reagents

Samples of raw milk were provided by LabUFMG, Escola de Veterinária, UFMG, in July 2009. The samples were obtained from dairy farms in accordance with recommended hygienic-sanitary conditions [24] and were stored at 4 °C for up to 5 days prior to analysis. The samples were used as a pool for the method optimization studies.

Samples of raw cow's milk and of some dairy beverages were purchased at local grocery stores. Human milk was provided by a human milk bank.

All reagents used were of analytical grade, except HPLC reagents which were LC grade. Ultrapure water was obtained from a Milli-Q System (Millipore Corp., Milford, MA, USA). The mobile phases were filtered through HAWP and HVWP membranes for aqueous and organic solvents, respectively (0.45 µm pore size, Millipore Corp., Milford, MA, USA).

Amines standards were purchased from Sigma Chemical Co. (St. Louis, MO, USA). They included spermidine trihydrochloride (SPD) and spermine tetrahydrochloride (SPM). *o*-Phthalaldehyde was also purchased from Sigma Chemical Co.

2.2. Screening of variables affecting polyamines extraction from milk

A Plackett–Burman design was used to screen the main factors that could affect recovery of amines from milk when using trichloroacetic acid (TCA) and sulfosalycilic acid (ASS). The design included 12 tests and three repetitions at the central point for each acid and five independent variables (Table 1). These variables were selected based on conditions used in previous studies [17,25] and also on limitations of the extraction procedure. The variables included were acid concentration, vortexing time, centrifugation speed, centrifugation time and number of successive extractions.

Table 1
Experimental values and coded levels of the independent variables used in a Plackett–Burman design to screen conditions for the analytical extraction of polyamines from milk with trichloroacetic acid (TCA) and sulfosalicylic acid (SSA).

Independent variables	Code units	Coded variable levels		
		−1	0	1
Acid concentration TCA/SSA (%)	X_1	5/4	10/12	15/20
Vortexing time (s)	X_2	30	60	90
Centrifugation speed (*g*)	X_3	6000	10,000	14,000
Centrifugation time (min)	X_4	5	10	15
Number of extractions	X_5	1	2	3

The temperature was fixed at 4 °C.

Ten-gram samples were incorporated with 0.6, 1.0, 1.2 and 1.6 mL of spermine and spermidine standard solutions at concentration of 25 µg mL^{-1}, to obtain samples spiked with different concentrations of the polyamines. The samples were homogenized thoroughly. A volume of acid was added to the sample and it was mixed by means of a vortex (Biomatic, São Paulo, SP, Brazil). The mixture was centrifuged at 4 °C in a refrigerated centrifuge (Jouan MR23I, Saint Herblain, France) [17,25,26]. When using SSA as the extracting acid, the samples were kept at 4 °C for 30 min prior to centrifugation [16]. The supernatant was collected and filtered through Whatman #1 and also through 0.45 µm HAWP membrane (Millipore Corp., Milford, MA, USA) prior to HPLC analysis. The results were reported as percent recoveries.

2.3. Optimization of the extraction using sulfosalicylic acid

Using the Plackett–Burman results, a Central Composite Rotational Design (CCRD) was conducted with three replications at the central point for SSA to optimize the recoveries of the polyamines. The variables considered were the number of successive extractions, vortexing time and concentration of SSA. The time and speed of centrifugation were set at 5 min and 14,000× g, respectively.

The experimental values and coded levels for the independent variables used in the CCRD are indicated in Table 2. According to rotatable conditions $[\alpha = (2^3)^{1/4}]$, α should be −1.68 and +1.68, but they were set at −2 and +2 because the number of extractions is a discrete variable. For the acid concentration, $-\alpha$ was equal to −1.3, which corresponded to 1.5% of SSA.

The statistically significant experimental results from the CCRD were fitted to models for spermidine and spermine with the coded independent variables. The optimized extraction conditions that provided the best recoveries were confirmed using four replicates. The determination coefficients (R^2) and the calculated (F_{cal}) and tabulated (F_{tab}) F values for the models proposed for each amine were also determined.

2.4. Validation of the optimized method

The fitness of the method for the determination of spermine and spermidine in milk was investigated by means of linearity,

Table 2
Experimental values and coded levels of the independent variables used in a Central Composite Rotational Design to determine the optimum conditions for the analytical extraction of polyamines from milk with sulfosalicylic acid (SSA).

Independent variables	Code units	Coded variable levels				
		−α[a](−1.68)	−1	0	+1	+α[a](+1.68)
Number of extractions	X_1	1	2	3	4	5
Vortexing time (seconds)	X_2	40	60	90	120	140
SSA concentration (%)	X_3	1.5	4	12	20	25

[a] α is different from ±1.68 for X_1 and from −1.68 for X_3.

Table 3
Screening of the factors that affect the recovery of spermidine (SPD) and spermine (SPM) during analytical extraction with trichloroacetic and sulfosalicylic acids using a Plackett–Burman design with five independent variables.

Design points	Independent variables[a]					Recovery (%)/extraction acid/polyamines			
						Trichloroacetic acid		Sulfosalicylic acid	
	X_1	X_2	X_3	X_4	X_5	SPD	SPM	SPD	SPM
1	1	−1	1	−1	−1	56.0	48.7	52.8	73.8
2	1	1	−1	1	−1	51.1	55.3	56.1	77.0
3	−1	1	1	−1	1	81.7	71.9	55.9	67.9
4	1	−1	1	1	−1	61.8	60.0	40.8	49.3
5	1	1	−1	1	1	81.5	67.6	69.2	75.7
6	1	1	1	−1	1	82.8	73.6	73.8	57.0
7	−1	1	1	1	−1	48.2	49.3	53.1	64.4
8	−1	−1	1	1	1	51.1	52.6	85.6	79.1
9	−1	−1	−1	1	1	82.3	67.6	59.6	65.0
10	1	−1	−1	−1	1	82.4	66.8	70.2	82.9
11	−1	1	−1	−1	−1	37.3	30.5	53.6	66.4
12	−1	−1	−1	−1	−1	59.0	63.4	46.1	47.2
13	0	0	0	0	0	69.1	64.8	71.1	83.7
14	0	0	0	0	0	70.5	55.4	56.5	76.8
15	0	0	0	0	0	68.1	53.7	62.9	80.6

[a] X_1 – acid concentration, X_2 – vortexing time, X_3 – centrifugation speed, X_4 – centrifugation time, and X_5 – number of extractions.

selectivity, accuracy, precision, detection and quantification limit [27]. Standard solutions were prepared at levels of 0.2, 0.4, 0.8, 1.6, 2.0, and 2.4 μg mL^{-1}.

2.5. Determination of polyamines in milk from different species and from dairy beverages

The optimized method was used for the analysis of polyamines from samples of raw cow's milk (13), dairy beverages (14) and human milk (5).

2.6. HPLC method for the determination of polyamines

The extracts were filtered through qualitative filter paper and HAWP membranes (13 mm diameter and 0.45 mm pore size, Millipore Corp., Milford, MA, USA). The amines were separated by ion-pair reverse phase HPLC and quantified fluorimetrically after post-column derivatization with *o*-phthalaldehyde (OPA), as described by Santos et al. [28].

Liquid chromatography was performed using a LC-20AD system connected to a RF-10AXL spectrofluorimetric detector at 340 and 450 nm of excitation and emission, respectively, to a CBM-20A controller and to a software (LC solution, Shimadzu, Kyoto, Japan). A reverse phase Nova-Pak® C_{18} column (300 × 3.9 mm id, 4 μm) was used with a Nova-Pak® C_{18} guard-pak insert (Waters, Milford, MA, USA). The mobile phases were as follows: A – solution of 0.2 mol L^{-1} sodium acetate and 15 mmol L^{-1} 1-octanesulfonic acid sodium salt, adjusted to pH 4.9 with acetic acid, and B – acetonitrile. The flow rate was set at 0.5 mL min^{-1} and the gradient was time (min)/%B: 0.01/15; 1/6; 16/26; 22/16; 26/33; 27/18; 30/38; 31.5/15; 68/15. The post-column derivatization reagent was delivered at 0.3 mL min^{-1}. It consisted of 1.5 mL Brij-35, 1.5 mL mercaptoethanol and 0.2 g OPA dissolved in a 500 mL solution of 25 g boric acid and 22 g KOH (pH adjusted to 10.5 with 3% KOH). The column and the post-column reaction apparatus were at 22 ± 1 °C. The identification of the amines was performed by comparison of the retention times of amines in samples with those of standard solutions and by adding the suspected amine to the sample. Amine levels were calculated by direct interpolation in an external calibration curves.

2.7. Statistical analysis

The Plackett–Burman and CCRD experiments were performed using STATISTICA 8.0 (Statsoft Inc., Tulsa, OK, USA). The quality of the fit of the second-order model equations was expressed by the coefficient of determination (R^2), and the statistical significance was determined by analysis of variance and F test ($p \leq 0.10$).

3. Results and discussion

3.1. Screening of variables affecting polyamines extraction from milk

The recoveries of the polyamines when using TCA (Table 3) varied from 37.3% to 82.8% for spermidine and 30.5% to 73.6% for spermine. Based on these results, satisfactory recoveries (>80%) were obtained for spermidine, although the recoveries for spermine were not acceptable [27].

During extraction with SSA (Table 3), the recoveries of the polyamines varied from 40.8% to 85.6% for spermidine and 47.2% to 83.7% for spermine. Therefore, satisfactory conditions were achieved for both amines when SSA was used as the extracting acid.

The results of the main effects of the Plackett–Burman design on spermidine and spermine extraction using TCA and SSA are indicated in Table 4. Among the independent variables investigated, the number of extractions (X_5) was the only statistically significant variable. When TCA was used, X_5 significantly affected the recovery

Table 4
Main effects in the recovery of amines during analytical extraction of spermidine and spermine from milk using trichloroacetic acid (TCA) and sulfosalicylic acid (SSA).

Independent variables	Mains effects/amines/extraction acids			
	Spermidine		Spermine	
	TCA	SSA	TCA	SSA
X_1	9.3	1.5	6.2	4.3
X_2	−1.7	1.2	−1.8	1.9
X_3	−2.0	1.2	0.8	−3.8
X_4	−3.9	2.0	−0.4	2.6
X_5	24.7	18.7	15.5	8.3
Average	65.5	60.5	58.8	69.8

X_1 – acid concentration, X_2 – vortexing time, X_3 – centrifugation speed, X_4 – centrifugation time and X_5 – number of extractions; bold when $p < 0.10$.

Table 5
Recovery of spermidine (SPD) and spermine (SPM) during analytical extraction from milk with sulfosalicylic acid using a Central Composite Rotatable Design.

Design point	Independent variables			Amines recovery (%)	
	X_1	X_2	X_3	SPD	SPM
1	−1	−1	−1	89.3	87.7
2	−1	−1	+1	80.5	97.3
3	−1	+1	−1	80.5	92.6
4	−1	+1	+1	75.3	87.5
5	+1	−1	−1	94.7	101.5
6	+1	−1	+1	86.1	93.1
7	+1	+1	−1	94.1	105.6
8	+1	+1	+1	85.5	106.6
9	−2	0	0	73.9	76.9
10	+2	0	0	88.3	106.0
11	0	−1.68	0	90.4	103.0
12	0	+1.68	0	89.2	89.4
13	0	0	−1.3	86.6	99.3
14	0	0	+1.68	88.4	101.5
15	0	0	0	84.6	100.0
16	0	0	0	89.5	106.8
17	0	0	0	87.0	108.4

X_1 – number of extractions; X_2 – vortexing time and X_3 – acid concentration.

of the amines ($p < 0.10$); however, when SSA was used, no significant effect was observed for spermine. Such a significance level was used to minimize the risk of excluding an important factor from the procedure in the following step.

SSA was selected as the extraction acid because it provided percent recoveries of the amines in accordance with recommended values [27]. Because the number of extractions significantly affected the recovery of the polyamines, they were further investigated in the subsequent design.

3.2. Optimization of the extraction using sulfosalicylic acid

The percent recoveries of the polyamines obtained in the CCRD are indicated in Table 5. Adequate recoveries were achieved under several experimental conditions. The coefficients of variation (CV) from the three replicates at the central point for all the responses were also adequate – below 5% (2.8% for spermidine and 4.2% for spermine), which is consistent with the limits established by Codex – 15% [27].

Based on these results, it was possible to build models for the extraction of spermidine and spermine using the coded independent variables that were statistically significant (Table 6). The coefficient of determination (R^2) and the F_{cal} and F_{tab} for the models are also indicated in Table 6. The R^2 and F_{cal} values for spermidine and spermine were lower than desired. However, the percentage of explained variance was appropriate for describing the phenomenon. The surface responses were built with the significant variables which lead to recoveries from 80% to 110% of the polyamines.

According to Table 6 and Fig. 1a, the recovery of spermidine was higher with an increased number of extractions and lower concentrations of the acid solution, whereas the vortexing time did not affect recovery. For spermine, according to Fig. 1b, an increase in the number of successive extractions and a decrease in the vortexing time provided higher recoveries, whereas acid concentration had no significant effect. Because an excessive number of extractions could dilute the extract and reduce the sensitivity of the method, four successive extractions were used thereafter. The optimum conditions for the extraction of the polyamines are four successive extractions, 40 s of vortexing and 1.5% SSA (Table 7).

Table 7
Extraction conditions providing a higher recovery's percentage of the amines.

Independent variables	Code units	Spermidine	Spermine
Number of extractions	X_1	+1 (4)	+1 (4)
Vortexing time	X_2	$-\alpha$ (40 s)	−1 (60 s)
Acid concentration	X_3	$-\alpha$ (1.5%)	Any

Table 8
Predicted and experimental recoveries of polyamines obtained in the optimum extraction conditions.

Amines	Recovery (%)	
	Experimental result[a] (CV)	Predicted result
Spermidine	92.8 (5.6)	93.0
Spermine	104.1 (5.8)	98.5

[a] Mean values from four replications. Optimum condition: 40 s vortexing, four successive extractions, SSA at a concentration of 1.5%, centrifugation for 5 min at 14,000 g. CV – coefficients of variation.

3.3. Validation of the method

The experimental results as well as the recoveries predicted from the models are indicated in Table 8. Recoveries from the four replicates were within the values recommended by Codex [27] – between 80% and 110%. Furthermore, the coefficients of variation were lower than 15% which was also in accordance with Codex [27]. Therefore, the models predicted the experimental values well, indicating that the strategy used was effective in the optimization of the method. It also indicated that the method was precise and accurate.

The optimized method was validated. The selectivity of the method was confirmed, e.g. there was good resolution among the peaks. The assumptions that the regression residues followed normal distribution and were homoscedastic and independent were confirmed. Significant regression and lack of significant linearity deviation ($p > 0.05$) indicated that the concentrations of polyamines from 0.2 μg mL^{-1} to 2.4 μg mL^{-1} were linear. The limits of detection and quantification of both polyamines were 0.2 μg mL^{-1} and 0.5 μg mL^{-1}, respectively.

3.4. Determination of polyamines in milk from different species and from dairy beverages

None of the samples analyzed (13 samples of raw milk and 14 samples of dairy beverages) contained detectable levels of polyamines. These results are similar to reported literature data [16,17,29] which suggests that cow's milk either raw or as dairy beverages is not a good source of polyamines. Only spermidine was found in the five human milk samples analyzed. It was detected at levels varying from nd (<0.5 μg mL^{-1}) to 0.84 μg mL^{-1}. Spermine was detected at trace levels. According to Pollack et al. [26], spermine is usually present in human milk. However, the spermine levels usually decrease during storage [15], which could explain the low levels of spermine detected in the samples analyzed.

Table 6
Coefficient of determination (R^2), $F_{cal,}$ F_{tab} and second-order coded model equations for the recovery of polyamines during analytical extraction under the conditions of CCRD.

Amines	R^2 (%)	F_{cal}	F_{tab}[a]	Second-order coded model equations
Spermidine	74.0	12.6	2.56	$\hat{Y} = 87.8 + 3.98\,X_1 - 1.76\,X_1{}^2 - 2.30\,X_3$
Spermine	70.4	10.3	2.56	$\hat{Y} = 102.8 + 6.3\,X_1 - 3\,X_1{}^2 - 2.7\,X_2{}^2$

[a] F_{cal} – calculated F value; F_{tab} – tabulated F value; $p \leq 0.10$.

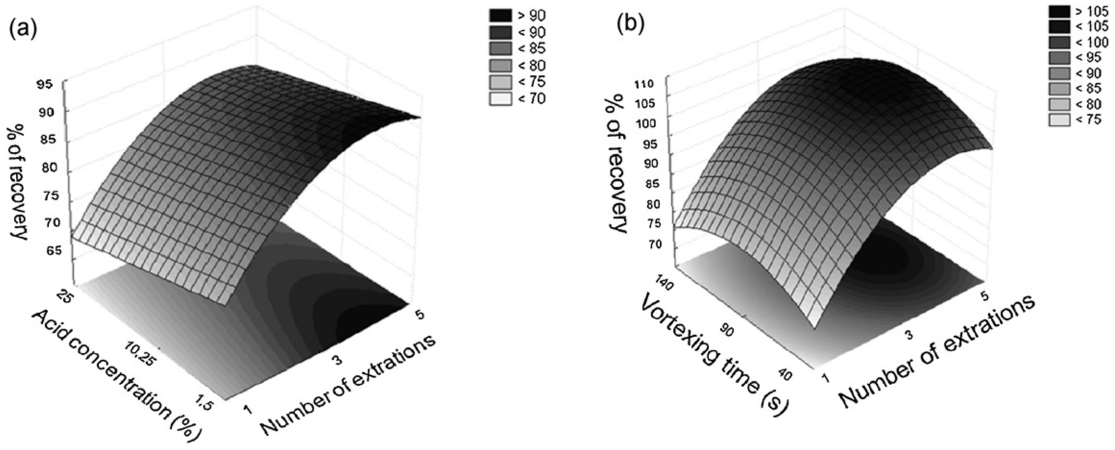

Fig. 1. Response surfaces for spermidine as a function of acid concentration and number of extractions (a) and for spermine as a function of vortexing time and number of extractions (b).

4. Conclusions

A method for the extraction of polyamines in milk was optimized. The variables that significantly affected the recoveries of the amines during extraction with TCA and SSA were screened through a Plackett–Burman design. SSA provided better recoveries than TCA. Sample centrifugation time and speed were independent variables. CCRD studies to optimize the dependent variables with SSA indicated that the optimum condition for the extraction of polyamines was 40 s vortexing, four successive extractions, and SSA at a concentration of 1.5%. These conditions provided recoveries $\geq$92.8% and CV $\leq$ 5.8%. Experiments confirmed the predicted results, indicating that the optimized conditions and models used were effective in the determination of polyamines from milk. Raw milk and milk beverages were shown to be poor sources of polyamines, whereas human milk can be a source of these amines. The optimized method can be used to further investigate the levels of polyamines in milk and the role of processing and storage.

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