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High-performance liquid chromatographic determination of polyamines in milk as their 9-fluorenylmethoxycarbonyl derivatives using a column-switching technique

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Abstract

A high-performance liquid chromatographic method for the determination of polyamines in milk is described. Polyamines were extracted in perchloric acid and derivatized with 9-fluorenylmethoxycarbonyl chloride (FMOC-Cl). The excess of reagent was reacted with aspartic acid before the analysis on a column-switching system. Linearity of derivatization was calculated for each amine and the coefficient of regression ranged from 0.994 to 0.999. Chromatographic separation of FMOC–polyamines was achieved with a gradient elution programme of water–acetonitrile. The correlation coefficients of the standard curves in the concentration range from 0.5 to 5 nmol ml^{-1} were higher than 0.991. The repeatability of the method, expressed as R.S.D. for each polyamines ranged from 3.0 to 8.6%. The percent mean recoveries at 1 nmol ml^{-1} spiking level were 49±3, 58±5, 61±5 and 48±4 for putrescine, cadaverine, spermidine and spermine, respectively. The limit of detection, calculated on the basis of three times signal-to-noise ratio, was 50 pmol ml^{-1} for each polyamine.
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1. Introduction

Natural polyamines (PAMs), putrescine (PUT), spermidine (SPD) and spermine (SPM), are essential constituents of most living organisms [1,2]. The concentration of these amines and their biosynthetic enzymes is high in actively proliferating animal tissues and increases rapidly when growth or differentiation is induced in resting cells; these changes usually precede increases in DNA, RNA and protein synthesis [3]. On the basis of these and other observations, it is generally believed that PAM biosynthesis is intimately interrelated with the synthesis of nucleic acids and proteins and that they are directly responsible for the increased macromolecular synthesis that occurs during growth and neoplasia [4–8].

The aim to clarify the biological function of PAMs in physiological and biochemical processes has led to the development of research on simpler and more sensitive analytical methods to quantify these compounds in different types of biological materials. Several methods have been reported in literature for the separation and determination of PAMs in various

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biological samples; ion-exchange chromatography (IEC) [9,10], thin-layer chromatography (TLC) [11–13], gas chromatography (GC) [14,15] and high-performance liquid chromatography (HPLC) [16–18] are techniques which have found a wide application in PAM assays.

TLC and IEC have been completely replaced by more sensitive and specific techniques. Pre-column derivatization followed by reversed-phase (RP) HPLC separation represents the preferred method applied for the separation and quantitation of PAMs.

Among the many suitable reagents, 5-dimethylaminophthalene-1-sulfonyl chloride (Dns-Cl) [11,19,20], benzoyl chloride [21,22], fluorescamine [23], orthophthalaldheyde (OPA)–2-mercaptoethanol [16,24] have been widely applied for prechromatographic derivatization of the PAMs. In contrast with the acid chlorides which form derivatives not only with primary and secondary amino groups, but also with phenols, amino acids and aliphatic alcohols, fluorescamine and OPA–2-mercaptoethanol are more selective reagents, reacting only with the primary amino group. On the other hand the derivatives formed by the latter two reagents are unstable, especially for spermidine and spermine containing untreated secondary amino groups.

More recently FMOC-Cl has been used in pre-column derivatization of amino acids [25] and PAMs [17]. This reagent reacts with primary and secondary amino groups forming stable and fluorescent derivatives. In a recent paper, Bartök et al. [18] reported a fully automated procedure for the determination of FMOC–PAMs in plant tissue.

Milk is one important source of PAMs for animals and babies [28]. It has been suggested that the content of PAMs in milk plays an essential role in postnatal growth of the mucosa of the gastrointestinal tract of the offspring [29].

In spite of the fact that a number of reliable methods for the separation of PAMs are available, more information should be achieved on the determination of PAMs in milk.

With the aim to introduce further improvements directed mainly to increase the sensitivity and the resolution of the technique, a method for the determination of PAMs in milk was developed in our laboratory, by using FMOC-Cl for their derivatization and a column-switching technique for the sample pre-concentration.

2. Experimental

2.1. Reagents and chemicals

Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, 1,7-diaminoheptane (DAH), 1,12-diaminododecane (DAD) and L-aspartic acid, were obtained from Sigma (Sigma, St Louis, MO, USA); 9-fluorenylmethyl-chloroformate was purchased from Fluka (Fluka, Buchs, Switzerland). Potassium borate solution 1.0 *M*, pH 10.4 was obtained from Pierce (Pierce, Rockford, IL, USA). Acetonitrile was of HPLC grade; all other solvents were of analytical-reagent grade and were from Merck (Darmstadt, Germany). Perchloric acid and hydrochloric acid were from Carlo Erba (Milan, Italy).

2.2. Standards

10 m*M* stock standard solutions of PUT, CAD, SPD, SPM, DAH and DAD were prepared in 0.2 *M* HCl and stored at −20°C. Working standard solutions at different concentrations were prepared from the stock solution by diluting with 0.2 *M* HCl.

2.3. Milk samples

Raw milk samples were obtained from Holstein Friesian cows between 100 and 200 days of lactation. Commercial milk samples were purchased from a local market.

2.4. Apparatus and chromatographic conditions

The isocratic HPLC system consisted of a Model 980-PU pump combined with a Model AS-950 autosampler (Jasco, Ishikawa-cho, Japan). Pre-concentration was carried out on a small stainless-steel column (50.0×4.6 mm I.D.) packed with LiChrosorb C_{18} stationary phase (particle size 10 μm). The PAMs were pre-concentrated with a water–acetonitrile (40:60, v/v) mixture at a flow-rate of 1 ml min^{-1}.

The valve switching system consisted of two Model 980-PU pumps (Jasco), combined with a

Model 7000 six-port column-switching valve (Rheodyne, Cotati, CA, USA). The analytical separation was achieved on a 250×4.6 mm I.D. stainless-steel column packed with Hypersil ODS material, particle size 5 μm (Shandon, Cheshire, UK). Gradient elution was performed from 100% of a water–acetonitrile (40:60, v/v) mixture to 100% acetonitrile in 20 min at a flow-rate of 1 ml min^{-1}.

Detection of the PAMs was performed on a Model 721-FP spectro-fluorimeter detector (Jasco), operated at the excitation and emission wavelengths of 264 nm and 313 nm, respectively. Detector signals were processed with an Hewlett-Packard Model 3365 Chemstation installed on a Hewlett-Packard Vectra VL2 personal computer and printed with a Deskjet printer (Hewlett-Packard, Avondale, PA, USA).

During injection (injection volume 50–100 μl) the two columns were positioned parallel to each other and the sample was injected on the first column (C1). After 6.5 min the valve was switched, the concentrated sample was transferred from the first to the second column (C2) and chromatographed with gradient elution. The valve was then switched back to the original position and the two columns were conditioned with a water–acetonitrile (40:60, v/v) mixture before further analysis. A schedule of the switching-column system is shown in Table 1.

2.5. Polyamine extraction

A 1 ml volume of milk was mixed with 200 μl of 10% perchloric acid solution in a Eppendorf tube and 100 μl of a 0.01 m*M* solution containing 1 n*M* of DAD as internal standard was added. The mixture was thoroughly vortexed and centrifuged at 10 410 *g* for 20 min with a Biofuge A centrifuge (Heraeus, Karlsruhe, Germany). The supernatant was transferred in another Eppendorf tube and 6–7 drops of 1.5 *M* KOH solution were added to adjust the pH to approximately 7.0. The mixture was centrifuged at 10 410 *g* for 20 min and the supernatant was collected in a screw-capped Pyrex glass tube. The solution was then defatted with a chloroform–methanol (2:1, v/v) mixture and the sample was centrifuged at 1306 *g* for 10 min with a Megafuge 1.0 R centrifuge (Heraeus). The aqueous phase was transferred to a screw-capped Pyrex glass tube.

2.6. Derivatization

A 50 μl volume of 1 *M* potassium borate pH 10.4 was added to the aqueous extract and thoroughly mixed to adjust the pH to 8.0. A 1 ml volume of 5 m*M* FMOC-Cl solution in acetonitrile was added to the basified aqueous extract and thoroughly mixed for 20 s.

After 60 s a 200 μl volume of a 100 m*M* aspartic acid solution in 0.01 *M* HCl was added to the reaction mixture. After 60 s the FMOC–PAM derivatives were extracted with 2 ml of *n*-pentane. The sample was centrifuged at 1306 *g* for 10 min and the organic phase transferred. The extraction in *n*-pentane was repeated and the organic phases combined. The organic solvent was evaporated to dryness under a stream of nitrogen. The dried residue was dissolved in 200 μl of the mobile phase. A volume of 50–100 μl of sample was injected on the column-switching system. The linearity of the derivatization was calculated in the concentration range from 0.5 to 5 n*M* of each polyamine.

Table 1
Schedule of the column-switching system

Time (min)	Event	Valve position	Eluent in C1	Eluent in C2
0	Inject	A	CH_3CN–water (60:40, v/v)	CH_3CN–water (60:40, v/v)
0–6.5	Load sample on C1	A	CH_3CN–water (60:40, v/v)	CH_3CN–water (60:40, v/v)
6.5	Switch valve; start integration	B	CH_3CN–water (60:40, v/v)	CH_3CN–water (60:40, v/v)
6.5–26.5	Gradient elution	B	From CH_3CN–water (60:40, v/v) to 100% CH_3CN	From CH_3CN–water (60:40, v/v) to 100% CH_3CN
26.5–37	Wash	B	CH_3CN	CH_3CN
34	Stop integration	B	CH_3CN	CH_3CN
37	Switch valve	A	CH_3CN	CH_3CN
37–42	Condition	A	CH_3CN–water (60:40, v/v)	CH_3CN–water (60:40, v/v)

C1=Column 1; C2=column 2.

2.7. Spiking studies

Recovery experiments were carried out on raw milk at 1 and 2 nmoles ml^{-1} spiking levels. Each amount was added to six replicates. The samples, including a raw sample, were submitted to the procedure described in Sections 2.5 and 2.6. To calculate the recovery of PAMs from spiked milk the concentrations determined before and after the addition of the standards were compared.

3. Results and discussion

The first approach to the development of the method was to perform a pre-column derivatization of the PAMs according to Price et al. [17].

To achieve almost complete derivatization of PAMs, an excess of FMOC at different reaction times (10 s, 30 s, 1 min, 5 min, 1 h) was used. The FMOC–PAMs derivatives were formed rapidly in 30 s and were stable at least for 1 h.

Unfortunately, when a portion of the derivatization mixture was injected directly after the reaction, many interference peaks resulted, especially when a $\lambda_{em}<$ 340 nm was used. This was expected, because the FMOC–PAMs, FMOC remaining after the reaction and the hydrolytic by-products of the FMOC have almost identical excitation and emission spectra. All these compounds, and probably some more hydrophobic FMOC–amino acids, were present in the chromatogram when spiked milk was analysed, disturbing the separation of PAMs, especially for PUT and CAD (chromatograms not shown).

To overcome this problem, a modification of the pre-column derivatization with FMOC described by Price et al. [17] was developed, based on the addition of aspartic acid to the reaction mixture. After the derivatization of the PAMs, the excess of FMOC was reacted with aspartic acid to form a FMOC–asp complex. This complex distributed into the aqueous phase, while the hydrophobic FMOC–PAMs were extracted with pentane. The addition of aspartic acid did not affect the reaction yield and minimized the formation of FMOC–alcohol and reaction by-products.

Subsequently, we developed a simple column-switching system that could be used for the concentration of the sample, allowing an injection volume of up to 200 μl.

As described in Section 2.4, during the injection of the sample the two column were positioned parallel to each other and the sample was injected onto the C1 column. Then the valve was switched, the concentrated sample was transferred from the C1 to the C2 column and chromatographed with gradient elution.

In order to determine the time necessary for the pre-concentration of the sample, the C1 column was directly coupled to the fluorescence detector, and the elution profile of PAMs with a water–acetonitrile (40:60, v/v) mixture at a flow-rate of 1 ml min^{-1} was registered. This experiment showed that 6.5 min was a sufficient time to remove most of matrix interferences without breakthrough of the PAMs. To monitor quantitative transfer of the PAMs to the C2 column 100 μl of a 0.01 m*M* standard solution was derivatized and analysed on the C2 column. The analysis of the same standard solution was then carried out in the column-switching system under the conditions described in Sections 2.4–2.6.

The experiment demonstrated that PAMs were quantitatively transferred from the C1 to the C2 column.

Chromatograms of a standard solution of PAMs after their derivatization with FMOC, spiked milk and raw milk samples are shown in Figs. 1–3, respectively. In the chromatograms, the 6.5 min corresponds to the switching of the valve and the

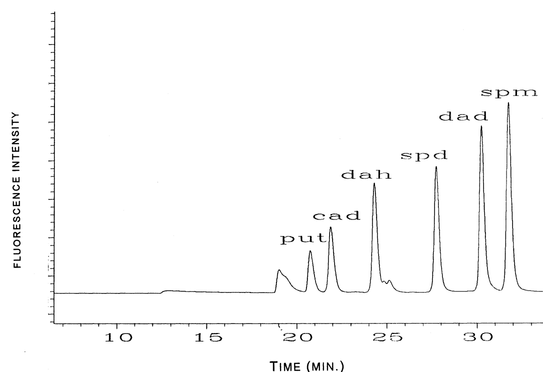

Fig. 1. Chromatogram of FMOC-derivatives of PUT, CAD, DAH, SPD, DAD and SPM standard (500 p*M* of each polyamine on-column).

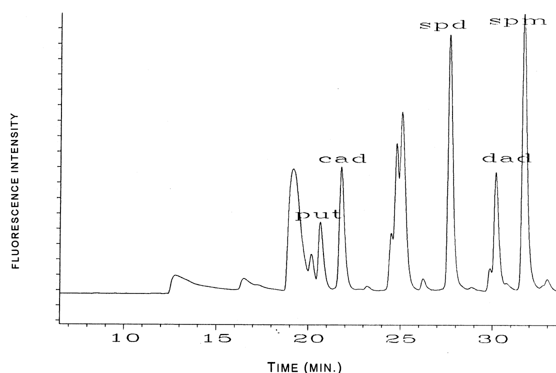

Fig. 2. Chromatogram of raw milk sample spiked with standard polyamines (2 nM of each amine added; 1 nM of 1,12 DAD as internal standard); 50 μl injected.

PAMs were eluted at 20.7, 21.9, 27.7 and 31.7 min for PUT, CAD, SPD and SPM, respectively. A fairly good separation of the peaks from interfering compounds was realized.

The data on the recovery and the repeatability of the method are shown in Table 2. The recovery of PAMs added in milk at 1 and 2 nmol ml^{-1} levels ranged from 48±4% to 65±2%. The within-laboratory reproducibility, expressed as relative standard deviation (R.S.D.), was 3.0 to 8.6%.

Linear calibration curves were obtained with known amounts of PAMs ranging from 0.5 to 5 nmol ml^{-1} (0.5, 1, 2, 3, 4, 5 nmol ml^{-1}), corresponding to the expected range of milk samples. The equations of these linear curves were found to be: FMOC–PUT, $y=45.6x+10.5$ ($r=0.991$); FMOC–CAD, $y=83.8x+20.5$ ($r=0.998$); FMOC–SPD, $y=128x+58.3$ ($r=0.994$); FMOC–SPM, $y=134.9x+32.6$ ($r=0.999$). The correlation coefficients (r) were found to be higher than 0.99.

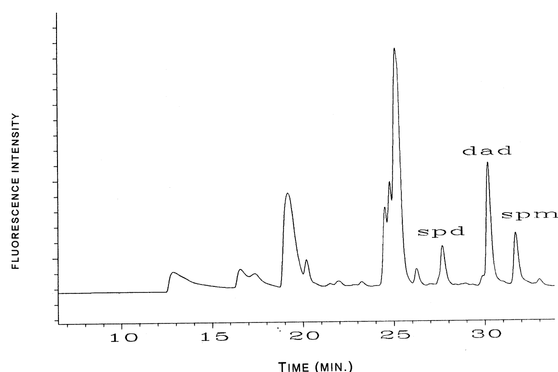

Fig. 3. Chromatogram of FMOC-polyamines in raw milk sample (1 nM of 1,12 DAD as internal standard); 50 μl injected.

Table 2
Recovery data obtained from raw milk spiked with standard polyamines

Compound	Recovery (%, mean±S.D., $n=6$)		Limit of detection (pmol ml^{-1})[a]
	1 nmol ml^{-1}	2 nmol ml^{-1}	
Putrescine	49±3	50±4	50
Cadaverine	58±5	56±3	50
Spermidine	61±5	65±2	50
Spermine	48±4	50±3	50

[a] Limit of detection was calculated on the basis of a signal-to-noise ratio of 3:1.

The lowest detectable amount of PAMs in milk was 50 pmol ml^{-1}, calculated as the amount of the polyamine which resulted in a peak area three-times greater than that of the baseline noise.

The overall recovery of the PAMs was rather low. This was probably due to the binding of the PAMs to the milk protein precipitate [26]. Nevertheless, the repeatability of the method, as demonstrated by the R.S.D.s, was satisfactory. Any attempts to improve the recovery at so low level of detection (0.1–2 nmol ml^{-1}) did not give attractive results. The effect of the pH during the defattening step was evaluated. No differences in extraction yield were noted when a pH lower than 7.0 was used, because the PAMs are already fully protonated at physiological pH [8]. Furthermore, other organic solvents, namely hexane, heptane, isooctane and toluene, were tested for the extraction of the FMOC–PAMs from the aqueous phase. Pentane showed the best recovery with less interfering substances and was used as the extracting solvent.

The use of DAH and DAD as internal standards was tested. Unfortunately, DAH was not well separated from unknown peaks having similar retention time. For this reason, DAH was abandoned and DAD was used as internal standard.

The applicability of the method has been demonstrated by the analyses of PAMs concentration of ten different samples of raw milk and ten different samples of commercial full cream milk. The concentration of SPD and SPM in raw milk ranged from

140 to 530 and from 340 to 1530 pmol ml^{-1}, respectively. In commercial milk the concentrations ranged from 130 to 490 pmol ml^{-1} for SPD and from 290 to 1210 pmol ml^{-1} for SPM. PUT and CAD were not detected. These data are in agreement with Motyl et al. [27], who found only traces of SPD and SPM in cow milk after the first month of lactation, and did not reported the presence of PUT and CAD. Reasonably this is due to a physiological decrease of PAMs content in late lactation. Furthermore, in commercial milk the low concentration of PAMs may be due to the activity of the milk polyamine oxidase, as described by Morgan and Toothill [30].

4. Conclusions

The described method allowed a very sensitive determination of PAMs in milk. With only 1 ml of milk and by handling a low amount of solvents, concentrations of 50 pmol ml^{-1} could be measured. Following a simple two-step extraction procedure the compounds were pre-concentrated and separated in a switching-column system and detected with a fluorescence detector. Further studies have been carried out in our laboratories to fully automatize the derivatization and analysis procedure.

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