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Original Article

Determination of Biologically Active Polyamines in Turkey Breast Meat by HPLC and Derivatization with Dansyl Chloride

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A B S T R A C T

Background and Objectives: polyamines (putrecsine, spermidine and spermine) are widespread components, which can be found in most of the foods like meat, fruits, vegetables, cheese and wine. Studies show that reducing the level of polyamines in cells may help to slow down some cancer processes; however, dietary polyamines may be required in wound healing, and for growth, maturation and regeneration of the intestinal mucosa. The aim of the present study is determination of biologically active polyamines by HPLC, and finding the best method of their derivatization.

Materials and Methods: Three different methods of derivatization and gradient elution were tested. Two wavelengths (225 and 254 nm) were applied to find the best way of detecting and analyzing the polyamines. The best method was chosen according to the good and sharp peaks.

Results: Results from different experiments suggested that the best condition for derivatization of polyamines is when the samples are put in warm water bath (40ºC) for nearly 1 hour. Additionally, acetonitrile (90%) and water HPLC-grade were chosen as mobile phases, and 254 nm was determined as the appropriate wavelength.

Conclusions: The proposed method and condition are good, fast and reliable that can be applied for analyzing and detecting the biologically active polyamines in turkey meat samples by HPLC.

Keywords: Polyamines, Turkey breast meat, Dansyl chloride, HPLC

Introduction

Dietary polyamines such as putrescine, spermidine and spermine are low molecular weight bases with an aliphatic structure (1). They have traditionally been classified within the group of biogenic amines (BAs). However, they started to be considered separately during the 1990s for their role in the growth and function of normal cells, and due to their mode of formation (2). The polyamines are ubiquitous, widespread from bacteria to mammals (3). They can be found in different food stuff like wine, fruits and vegetables, cheese and meat in low concentration (4). These components have positive and negative effects on human health; they can be useful for growth and wounds healing, and also harmful for those suffering from tumor as they accelerate the growth of tumors (5).

Different methods have been used for measuring the biogenic amines (BAs) and polyamines existing in foods. Gas chromatography (GC) (6), thin-layer chromatography (TLC) (7), capillary electrophoretic (CE) (8) method, ion chromatography mass spectrometry (9), ultra-performance liquid chromatography (UPLC) (10) and high performance liquid chromatography (HPLC) are techniques that have been applied ever to determination BAs and polyamines (11). HPLC is the most widely used technique for its high sensitivity and wide range of linearity (12). GC is rarely applied for determination of BAs. Although TCL is a good method that does not need complicated equipment; however, it is time consuming with low sensitivity, so it has not been applied mostly. Additionally, CE is not a good technique in detecting BAs as they do not exhibit strong fluorescence, so they could not be detected directly in a sensitive manner. Among the mentioned methods, HPLC with pre- or post-column derivatization is by far the

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most frequently reported technique for BAs separation and quantification (11). Extraction of BAs is so important because most of them are linked to other components. Due to complexity of the meat matrices (protein-rich food, often with high-fat content) and low amount of BAs and polyamines in different matrices, extraction and purification of these components are necessary for analyzing. Indeed, acid extraction is mostly used for these components by hydrochloric acid, perchloric acid or trichloroacetic acid (TCA)(13).

As mentioned, BAs and polyamines do not have chromophoric or fluorogenic properties, so they are determined by pre-column or post-column derivatization (13). Derivitisation procedure is so crucial that should be done precisely. Some reagents that are mostly used for derivatization include dansyl chloride (14), benzoyl chloride (13) and orto-phthaldialdehyde (OPA) (15). Among these reagents, dansyl chloride is frequently used specially for spermidine and spermine. Smela et al. (2003) compared dansyl chloride and orto-phthaldialdehyde (OPA), and concluded that dansyl chloride is more suitable for derivatization of spermidine and spermine (16).

In the present study, we determined the polyamines in turkey breast meat by HPLC. Dansyl chloride was chosen for derivitisation, and the best condition was optimized. Furthermore, different gradient elutions and wavelengths were tested to find the best one that can give sharp and separate peaks in the chromatograms.

Materials and Methods

Reagents, materials and standards: Polyamine standards including putrescine dihydrochloride, spermidine trihydrochlride, spermine tetrahydrochloride and derivative reagent dansyl chloride were obtained from Sigma-Alderich. Acetonitrile, methanol, acetone and water (HPLC-grade) were purchased from Dae Jung (South Korea). Hydrochloric acid (HCl) (37%, w/w), sodium hydroxide (NaOH), and ammonia were purchased from Merck (Darmstadt, Germany).

The mixed stock standard solutions were prepared at a concentration of 2000 µg mL^{-1} in HPLC-grade water. The stock standard solutions were diluted with HPLC-grade water to obtain a working solution (100 µg mL^{-1}). Stock and working solutions were stored in darkness at 4 ºC in a refrigerator.

Instrument: A high performance liquid chromatography system Cecil CE-4900 (Cambridge, England), which consisted of two CE-4100 pumps, multiple solvent delivery unit, vacuum degasser, mixing chamber, six-port valve (Rheodyne, USA), and CE-4200 UV-Vis detector (Cambridge, England), was used for chromatographic analysis. An ODS column (250 mm×4 I.D., particle size 5µm) was used for separation of the analytes.

Sample preparation: The turkey breast meat samples were minced with a kitchen blender to achieve a homogeneous sample. 100 g of the ground sample was spiked (5 µg mL^{-1}), and the spiked samples were homogenized and stirred vigorously. 8 mL of perchloric acid (1mol l^{-1}) was added to 1 g of the spiked samples. Afterwards, the mixture was transferred into the centrifuge tube, and centrifuged at 4000 rpm for 10 min. Then the aqueous phase was transferred to another vessel, and Carrez was added in order to denature proteins. Next, it was centrifuged again at 4000 rpm for 10 min. Finally, polyamines were extracted and kept until use for derivatization.

Derivitization: As mentioned, to detect and separate polyamines, derivatization is necessary. Three different derivatization procedures were applied to find the appropriate one. In all procedures, dansyl chloride (5 mg/ml^{-1} acetone) was used. Additionally, derivitisation was done in darkness as it is so sensitive to light.

Method 1: One mL of mix or single standard was transferred to the test-tubes, and their pH was adjusted to 8 with 2M NaOH. Then 1 mL of dansyl chloride was added, and the mixture was shaken in darkness at laboratory temperature for 20 hours. Afterwards, 250 µL of L-prolin (0.1 g per 1 ml of water) was added to remove an excess of dansyl chloride. The samples were shaken gently for 1 more hour. Finally, the samples are ready to inject to HPLC.

Method 2: This method was similar to the previous one except in time and temperature. In fact, to accelerate the derivitisation procedure, the temperature was increased up to 60ºC and also the time of procedure was reduced to 2 hours.

Method 3: In this method, pH of the samples was adjusted to 11 with 2M NaOH, and 1 mL of fresh dansyle chloride was added immediately. Then the samples were transferred to warm water bath for 45-60 minutes while the temperature was set on 40ºC, and the samples were being shaken occasionally. Afterwards, 250µL of ammonia was added to eliminate extra derivative reagent. Eventually, the analytes were ready to inject to HPLC for detection and analysis.

Gradient elutions

Gradient elution program 1: Different programs were tested aiming to find sharper peaks with good retention time in the HPLC runs. Acetonitrile is a solvent that is mostly used to wash the column of HPLC and separate the analytes. In this case, acetonitrile 90% (90% acetonitrile + 10% HPLC-grade water) was applied for pump A, and methanol 90% was used for pump B. Wavelength was set on 225 nm. Gradient elution was as shown in table 1.

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Table 1. Gradient elution: Program 1

Time (min)	Pump A (Acetonitrile) %	Pump B (Methanol) %	Flow Rate (mL min^{-1})
0	100	0	0.8
17	100	0	0.8
19	0	100	0.8
26	0	100	0.8
28	100	0	0.8
40	100	0	0.8

Gradient elution program 2: The second gradient elution program is shown in Table 2. Acetonitrile was applied for pump A but HPLC-grade water was used in pump B. Wavelength of 254 nm was selected in this condition.

Table 2. Gradient elution: Program 2

Time (min)	Pump A (Acetonitrile) %	Pump B (Water) %	Flow Rate (mL min^{-1})
0	75	25	0.8
1	75	25	0.8
10	90	10	0.8
12	100	0	0.8
16	100	0	0.8
23	100	0	0.8
40	75	25	0.8

Gradient elution program 3: The gradient elution and the percentage of acetonitrile were changed in this step (Table 3). Acetonitrile 90% (90% acetonitrile + 10% HPLC-grade water) was used in this program. Mobile phase and wavelength are similar to the previous program.

Table 3. Gradient elution: Program 3

Time (min)	Pump A (Acetonitrile)%	Pump B (Water)%	Flow Rate (mL min^{-1})
0	75	25	0.8
1	75	25	0.8
10	90	10	0.8
12	100	0	0.8
16	100	0	0.8
23	100	0	0.8
33	100	0	0.8
34	75	25	0.8
40	75	25	0.8

Figure of merits: To estimate the performance of the proposed method, linearity, recovery, repeatability, limit of detection and limit of quantification were studied (table 4). Good linearity was obtained in range of 1-100 µg g^{-1} (ppm) for all analytes. Also the repeatability of putrecsine, spermidine and spermine determination was obtained as 7.30, 6.88 and 6.72, respectively. Recovery of polyamines was assessed by adding 2 µg g^{-1} (ppm) of the polyamine standards to the samples. Indeed, the recovery index was 105% for putrecine, 95% for spermidine and 80% for spermine. Additionally, the detection and quantification limits were 0.91 and 3, 0.7 and 2.3 and 0.53 and 1.7 ng g^{-1} for putrecsine, spermidine and spermine, respectively. However, SPD contents were below the detection limits (Table 5).

Table 4. Figures of merit for turkey breast meat samples

Analyte	R^2	RSD (%)	Recovery (%)	LOD (ng g^{-1})	LOQ (ng g^{-1})
Putrecine	0.98	7.30	105	0.91	3
Spermidine	0.97	6.88	95	0.70	2.3
Spermine	0.98	6.72	80	0.53	1.7

Table 5. Analytical results of polyamines in turkey breast meat samples

Sample	PUT (µg g^{-1})	SPD (µg g^{-1})	SPM (µg g^{-1})
1	9.71±0.61[a]	ND[b]	19.50±0.85
2	5.95±0.42	ND	15.33±1.05
3	9.71±0.33	ND	19.55±0.71
4	19.80±0.95	ND	38.03±1.30
5	3.27±0.25	ND	7.87±0.65
6	3.15±0.10	ND	6.55±0.55
7	3.39±0.10	ND	6.81±0.22
8	9.21±0.42	ND	17.20±0.48
9	17.53±0.51	ND	34.48±0.75
10	4.35±0.25	ND	7.43±0.35

a: Mean value ± standard deviation (n = 3).
b: Not detected

Results

According to the results and chromatograms, the third method is the best way to derivatate polyamines. The most important factor in derivatization is the pH of samples. Our results revealed that pH=11 is the optimum pH for this step and there was no acceptable chromatogram when pH is low. Indeed, derivatization process is not ideal when pH decreases. Another important factor is the temperature of derivatization. It is clear that temperature can increase the rate of any reaction. Temperature can influence derivatization too, so two temperatures of 40ºC and 60ºC were examined. The results showed that the more temperature increases, the more derivative components decompose because they are sensitive to high temperature, and will destroy when the temperature increases. Thus, lower temperature (40ºC) is more acceptable. Additionally, the freshness of dansyl chloride is important and it is better to make it weekly.

For gradient elution, it is distinguished that to separate analytes in the column, acetonitrile was the best solvent. It also gives good chromatograms. Firstly, it was assumed that polyamines have memory effects in the column, so methanol was used to wash the column and make it ready for the next injection. Finally, it was revealed that acetonitrile and water can remove the residuals completely. The results showed that the third gradient elution separates the analytes perfectly, and there was no interference of the peaks, and the retention time of each

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polyamine was suitable. In other conditions, the peaks overlapped and were close to each other. Suitable wavelength to detect polyamines was found to be 254nm as sharper peaks are seen in this wavelength (Figure 1).

The proposed method comparing to other methods is very suitable with high repeatability and appropriate detection of limit that could be applied in other laboratories. Indeed, preparation of samples in this method is easy and fast. In addition, the chromatograms achieved by this method showed no matrix interference, so it can be applied to other meat samples and also other matrices.

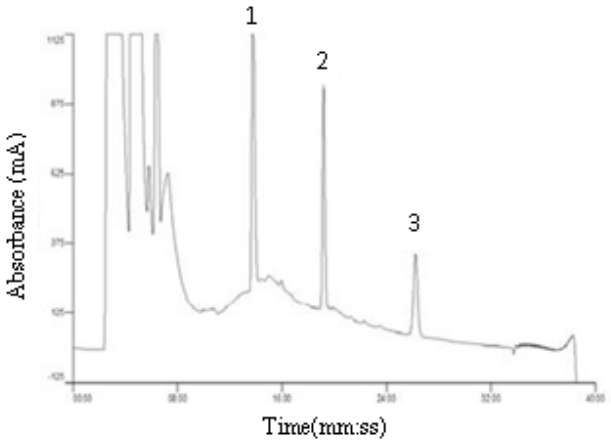


Fig 1. HPLC chromatograms of the mixture of standard biogenic polyamines (10 µg ml^{-1}) derivatized according to Method 3, gradient elution 3 and wavelength 254 nm. . Peaks identification: 1) putrescine; 2) spermidine and 3) spermine (14-27 min).

Discussion

According to our results and comparison to other studies, derivitisation is more desirable when the pH of samples is high. The optimum pH for derivitisation is considered by several researchers, but the results are not the same. For example, Innocent et al. (2007) reported that pH=11 is ideal that confirms our findings (17). On the other hand, Krausova et al. (2008) studied the changes of polyamines during the storage period and culinary treatments on pork lion, and introduced low pH for derivitisation. Indeed, they reported pH=8 is the optimum pH for the procedure by dansyl chloride (14) that is not consistent with our findings. Additionally, Smela et al. (2003) compared two derivative reagents, and concluded that dansyle chloride had more reliable chromatograms to OPA (16). Some important factors in derivitisation have been studied by several researchers. Krausova et al. (2008) reported room temperature for 20 hours as good point for derivitisation (14) while Innocent applied 40ºC for 1 hour (17); the latter confirms our findings. Comparison between different wavelengths (225 nm and 254 nm) showed that higher wavelengths introduces better and sharper peaks, so most of the researchers used 254 nm in their studies (11, 18). Moreover, different gradient elutions were applied by researchers; each has their own properties. However, in our study, acetonitrile and water were suitable as mobile phase that offered great retention time for each polyamine. According to our findings, retention time for putresine, spermidine and spermine is 12-13, 17-18 and 25-26 minutes, respectively. Use of acetonitrile and water as mobile phase is seen in several studies (19), yet some other solvents like methanol is seen too (20).

To summarize, use of dansyl chloride is a good choice to derivatate polyamines if the optimum conditions are applied: pH=11, temperature= 40ºC for 1 hour, acetonitrile 90% as mobile phase for pump A, and water HPLC-grade for pump B with gradient elution, and the wave length of 254 (Table 3). By applying the above conditions, good chromatograms with sharp and separate peaks are achievable.

Real sample analysis: Some sample were purchased from Tehran market, and the tested with proposed method to evaluate the reliability of the developed technique. The concentrations of the analytes were calculated from the linear regression equations of the standard curves. The results are shown in table 5, A clean separation and good chromatogram is readily achieved without the presence of matrix interference (Fig. 2).

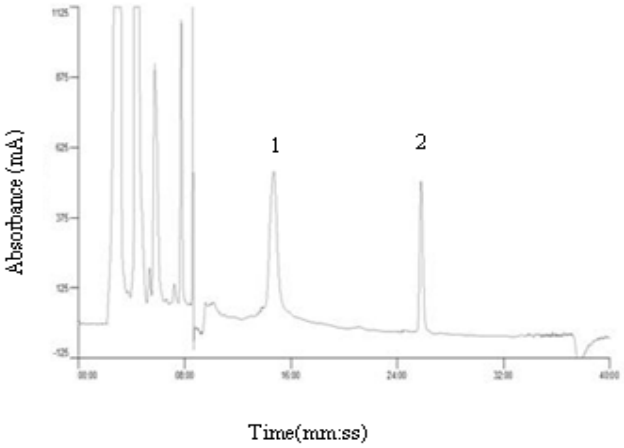


Fig 2. HPLC chromatogram of polyamines in turkey breast meat samples [1) PUT=15 min, 2)SPM=25 min]

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