

THE DETERMINATION OF PYRIMIDIN BASES IN CHICKEN EGG'S YELLOW BY HPLC

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Tavuk Yumurta Sarısında Pirimidin Bazlarının HPLC ile Tayini

Özet : 20°C ve 70°C'de tavuk yumurtası sarısında urasil, timin ve sitozinin perklorik asit ile ekstraksiyonu HCl ve H₂SO₄ den daha iyi çöktelti verdi. Pirimidin bazları ODS kolonunda 0.3 M NaH₂SO₄ mobil fazı ile ayrıldı. 261 nm de tanınan kromatogramlar daha ileri temizliğe gerek olmadığını gösterdi.

Serbest ortalama urasil konsantrasyonu 20°C de 49.89 µg/ml ± 20.03 ve 70°C'de 324 µg/ml ± 31.04 . Sitozinin (18.96 µg/ml ± 18.40 SE) ve timinin (3.73 µg/ml ± 3.45 SE) serbest ortalaması 20°C'de tavuk yumurtası sarısında güvenilir olmayacak kadar değişkendir. Geri almada, sitozin ve urasil eklenmiş yumurta sarısının 70°C asitle muamelesi bu baz konsantrasyonlarını artırması önemli görünmektedir (P<0.05). Yüksek sıcaklığın timin üzerine bir etkisi yoktur.

Yumurta sarısının serbest urasil konsantrasyonu güvenilir bir şekilde ölçülebilir. Sıcaklığın asitle ekstraksiyon üzerine etkisinin, tavuk yumurta sarısında sitozin kadar urasilinde farklı şekillerde (nükleosit, nükleotit) bulunabileceğini gösterdi. Sitozin, urasil ve timinin geri alma oranları % 96-150, % 77-180 ve % 56-67 dir.

Anahtar Kelimeler: Yumurta, Pirimidin, HPLC.

Summary: Perchloric acid extraction of uracil, thymine and cytosine in chicken egg's yellow at 20°C and 70°C gave the better precipitate than HCl and H₂SO₄ . Pyrimidine bases were separated on an ODS colon with 0.3 M. NaH₂PO₄ as mobile phase. Chromatograms detected at 261 nm indicate no further cleanup required.

Free mean uracil concentration was 49.89 µg/ml ±20.03 at 20°C and was 324.73 µg/ml ±31.04 54.0 at 70°C. Free mean of cytosine (18,96 µg/ml ±18.40 SE) and thymine (3.73 µg/ml ±3.45 SE) in chicken egg's yellow at 20 °C seem too much variable to be reliable. In recovery, 70°C treatment during acid extraction of egg yellow added with cytosine and uracil seems to increase significantly (p<0.05) these base concentration. High temperature has no effect on added thymine.

Free uracil concentration of chicken egg can be reliable measured. Temperature effect on acid extraction indicated that uracil as well as cytosine might be in different forms (nucleoside, nucleotide) in chicken egg's yellow. Recovery percentages of cytosine, uracil and thymine are 96-150 %, 77-180 % and 56-67 %, respectively.

Key Words: Egg ,Pyrimidine, HPLC

Introduction

Uracil, thymine and cytosine are very well known pyrimidin bases which give a maximum absorption 260, 265 and 276 nm., respectively (2, 10) . In animal tissue, they are mainly contributed in DNA and RNA structure (2). Determination of these free bases and bound bases through nitrogen analysis are known but these analysis are more tedious and unreliable because of the possibility of nitrogen loss.

The most challenging part is the liberation of these bases from nucleic acid. In general, acid-labile glucosidic bonds are cleaved with HClO₄ that is better than HCl and H₂SO₄ under heat. Base composition of DNA by 88% formic acid was also reported under 175 °C for 30

minutes (2). Hydrolysis of ribopolynucleotides by 1-0.3 N sodium or potassium hydroxide takes longer time (16 to 24 hours). Phosphodiester bonds, N-glycoside linkages of purine groups are very sensitive to acid hydrolysis (15). Liberation of pyrimidin groups of nucleic acids depends on drastic conditions. Cytosine and Uracil can be recovered after heating with 12 N HClO₄ at 100 °C for 1 hour. Destruction of cytosine with N. HCl, H₂SO₄ and concentrated formic acid had been reported (15).

Preliminary separation of pyrimidin bases is achieved by paper chromatography (8). Usually ion exchange chromatography finds acceptability (4, 9, 16). Separation of nucleic acid bases with resin coupled with 8% thymine (affinity chromatography) containing colon reported to give good results (6). It has been reported that ion-

exchange column gives good results, but they are not stable over long periods. Reverse phase columns gives better reproducible results and shorter elution time (11). Recently, separation of nucleic acid bases and their derivatives with C8 (14) and C18 (5, 7, 11, 17, 18) column has found more acceptability.

This paper describes the determination of pyrimidin bases in chicken egg's yellow by HPLC, which for we have not come across any report.

Material and Methods

Reagents

(a) *Uracil*-(pfs)(2,4-dihydroxypyrimidine). Crystalline (Sigma Chemical Co., St. Louis, M.

(b) *Thymine*-(pfs)(2,4-dihydroxy-5-methylpyrimidine, 5-methyluracil). Crystalline (Sigma Chemical Co., St. Louis, MO)

(c) Cytosine-(pfs) (4-amino-2-hydroxypyrimidine). Crystalline (Sigma Chemical Co., St. Louis, MO)

(d) Perchloric acid.- 60 % (ACS) (E. Merck, Darmstadt, Germany)

(e) Sodium dihydrogen phosphate dihydrate.- (usp) (E. Merck, Darmstadt, Germany)

(f) Mobile phase.- Mobile phase was 0.3 M. NaH_2PO_4 prepared by double distill water and pH adjusted to 3 with a glass electrode. On a daily basis before use, the mobile phase was filtered (Millipore, 0.45 mm) and degassed under vacuum.

(g) *Preparation of standard solutions*.- Stock pyrimidine standards were prepared by dissolving 120 mg thymine, 120 mg uracil and 120 mg cytosine in 0.02 M KH_2PO_4 (pH=3.56) and filled up to 50 ml. Final

mixture of 5 ml cytosine + 5 ml uracil + 5 ml thymine was used for the standard calibration. This mixture has given to reach 12 mg of each bases for each extraction.

Apparatus

(a) *Glassware*.- All glassware was taken from original box and cleaned with acid (HCl), washed with distilled water until acidity was removed. Finally glassware was rinsed with ethanol and dried at 100 °C.

(b) *Chromatography*.- A Model LC-10AD HPLC pump (Shimadzu, Japan) was used to deliver the mobile phase isocratically at a flow rate of 1 ml/min. Samples were injected through Rheodyne 7124 injection valve (fitted with 20- μl loop). Pye Unicom 4020 model UV detector (Philips) was operated at 261 nm. Samples were separated by ODS colon (150x4.6 mm, Shimadzu, Japan) with ODS guard colon at ambient temperature. Results were calculated by C-R6A model chromapac integrator (Shimadzu). Heraeus Sepatech model refrigerated centrifuge has been used to spin precipitates.

Procedure

Extraction.- After Daily chicken eggs' yellows were separated from yolk gently, extensive yolks were dried out on filter paper without damaging yellow membrane. Egg yellows were weighted in 250 ml flasks with their membrane. To precipitate proteins, 30 % dilution of HClO_4 was used. Precipitates were filtered over Whatman (#42) filter paper. Filtrates were centrifuged at 6000 rpm for 30 minutes. Clear supernatants were introduced in column. Detailed extraction procedures were given at table 1.

Table 1. Extraction steps (Ex) of egg yellow are given below.

Steps	Ex-1	Ex-2	Ex-3	Ex-4	Ex-1	Ex-2	Ex-3	Ex-4
1	egg yellow	egg yellow	egg yellow	water	egg yellow	egg yellow	egg yellow	water
2	20 ml HClO_4	20 ml HClO_4	15 ml standard mixture	15 ml standard mixture	20 ml HClO_4	20 ml HClO_4	15 ml standard mixture	15 ml standard mixture
3	5 minutes shake							
4	15 ml standard mixture	15 ml dd. water	20 ml HClO_4	20 ml HClO_4	15 ml standard mixture	15 ml dd. water	20 ml HClO_4	20 ml HClO_4
5	1 h at 20 °C				1 h at 70 °C			
6	Read at 261 nm UV detector							

Results and Discussion

Perchloric acid extraction of uracil, thymine and cytosine in chicken's egg yellow at 20°C and 70 °C gave the better precipitate than HCl and H₂SO₄. Full separation chromatogram of standard concentration of cytosine, uracil and thymine on ODS colon was shown in figure 1. Detection of pyrimidine from extraction 1 was given in figure 2 and detection of pyrimidine from extraction 4 were shown in figure 3. Retention times for cytosine, uracil and thymine were 1.81, 2.41 and 5.64 minutes, respectively. This indicated that good separation has been achieved. Extraction was designed to find out whether the acid or temperature has any effects on pyrimidine (Table 1). Data were processed based on a GLM procedure by a personal computer program of SAS (13) and the results were summarized in figure 4.

Concentration of uracil in egg yellow at 20 °C and 70 °C indicates the presence of free and bounded uracil. However, free cytosine and thymine levels in chicken egg's yellow at 20 °C have a wide variability with low concentration. Acid and temperature can easily cleave nucleoside and nucleotides (2, 15). Duncan's statistical test (3, 13.) suggests that treatment of cytosine and uracil with high temperature (70 °C) significantly increased ($P<0.05$) these pyrimidine quantities. High temperature had no effect on thymine concentration. When interaction between temperature and extraction procedures were examined, this interaction has no effect on the amount of thymine and cytosine, but uracil concentration ($P<0.005$). Table 2 illustrates addition order of acid or standard seems to have no effect on pyrimidine concentration.

Detailed examination of figure 4 indicated that temperature somehow increased amount of cytosine and uracil. This increase might be due to cleavage of nucleotid or nucleotides. However cytosine concentration seems too labile to temperature. Specially examination of uracil indicates that temperature treatment significantly increased egg yellow uracil concentration while did not significantly effect cytosine and thymine (Extraction 2).

For each extraction step 300 µg cytosine, 300 µg/ml uracil and 300 µg/ml thymine added. Recoveries from these additions were given in table 3. Under high molar acid (12N perchloric acid) and temperature (100 °C), good recovery of cytosine and uracil, but destruction of cytosine with sulfuric, hydrochloric and formic acid has been reported (15). Relative recovery percentage of cytosine, uracil and thymine has found among 96-157%, 77-180% and 56-67, respectively.

Dietary source of nucleotides has recently become very important (1, 12). Therefore it is necessary to know available nucleotide base concentration of chicken egg. Extraction and determination of pyrimidin bases in chicken egg yellow have been achieved by means of

acid extraction steps and HPLC rapidly. Recovery values indicate that temperature might increase cleavage of nucleoside or nucleotide to free base amount. Purin base levels have also be under investigation yet.

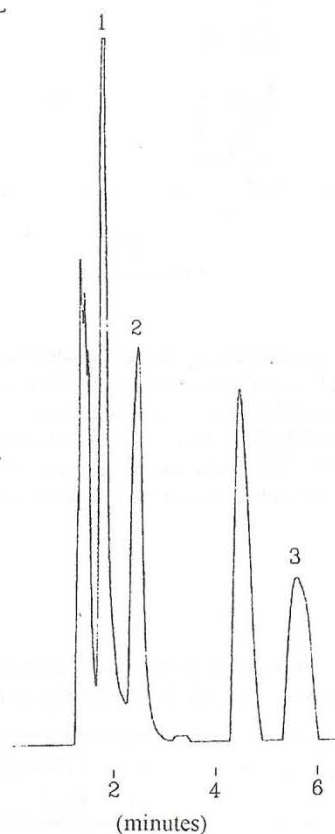


Figure 1. Full chromatogram of standard concentration of cytosine¹, uracil² and thymine³ separated on ODS column (150x4.6mm). Mobile phase was 0.3 M NaH₂PO₄ (pH=3.0). Detection was made at 261 nm UV. Retention times of cytosine, uracil and thymine were 1.81, 2.41 and 5.64 minutes, respectively.

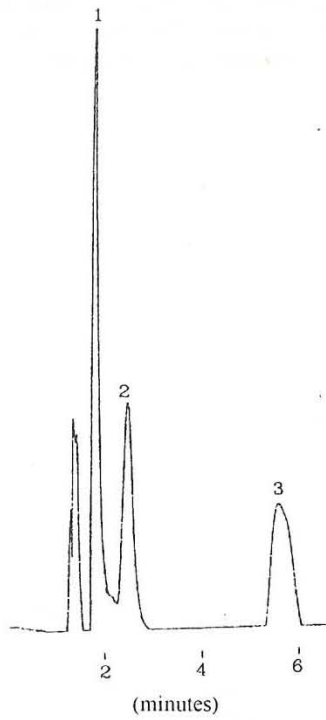


Figure 2. Full chromatogram of standard concentration of cytosine¹, uracil² and thymine³ from extraction 1 separated on ODS column (150x4.6mm). Mobile phase was 0.3 M NaH₂PO₄ (pH=3.0). Detection was made at 261 nm UV. Retention times of cytosine, uracil and thymine were 1.81, 2.41 and 5.64 minutes, respectively.

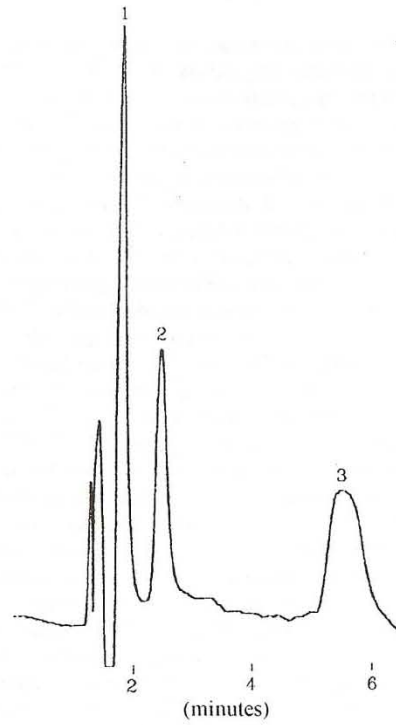


Figure 3. Full chromatogram of standard concentration of cytosine¹, uracil² and thymine³ from extraction 4 separated on ODS column (150x4.6mm). Mobile phase was 0.3 M NaH₂PO₄ (pH=3.0). Detection was made at 261 nm UV. Retention times of cytosine, uracil and thymine were 1.81, 2.41 and 5.64 minutes, respectively.

Table 2: Pyrimidine levels of different extraction procedures Mean (X) values within the same temperature level with different letters , differs significantly at P<0.05 levels"

Extraction	n	Cytosine mean (X) (µg/ml) ± SE	Uracil mean (X)(µg/ml) ± SE	Thymine mean (X)(µg/ml)±SE
1	12	355.11 ^a ± 43.35	397.32 ^a ± 50.56	192.34 ^a ± 7.88
2	6	9.48 ^b ± 9.26	187.31 ^b ± 63.52	3.7 ^b ± 2.10
3	6	389.18 ^a ± 43.40	404.63 ^a ± 64.13	173.72 ^a ± 8.55
4	6	317.28 ^a ± 15.09	230.15 ^b ± 25.23	191.72 ^a ± 11.34

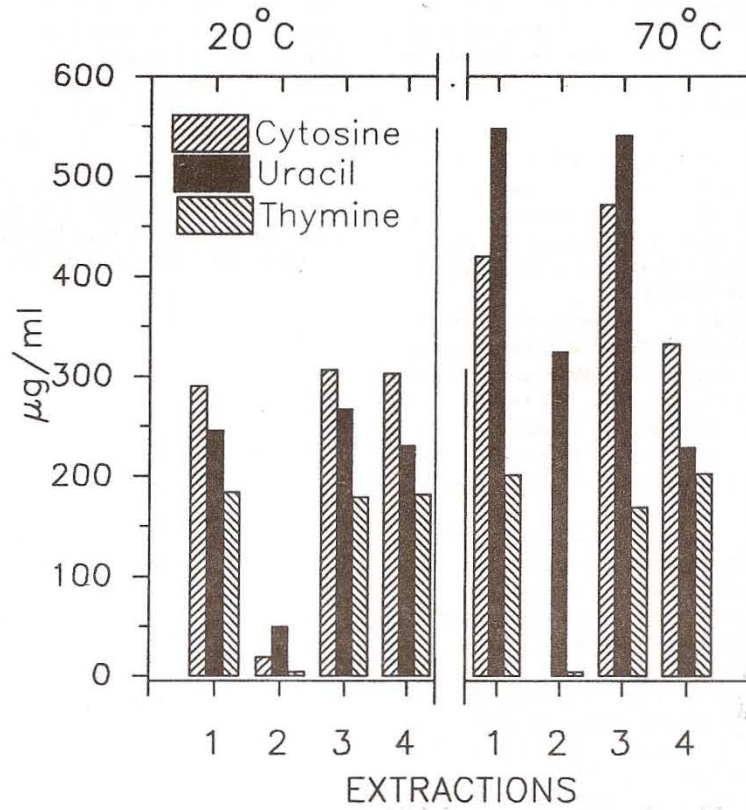


Figure 4: Pyrimidine means levels in chicken egg's yellows with different extraction procedure and temperatures. Extraction 1 and 3 are egg yellow spiked with pyrimidine. Extraction 2 is pyrimidine levels of egg yellow. Extraction 4 is pyrimidine levels from control.

Table 3: Recovery values of pyrimidin bases in egg yellow for each extraction steps

Temperature (°C)	Extraction	n	Relative recovery of cytosine %	Relative recovery of uracil %	Relative recovery of thymine %
20	1	6	96.74	82.14	61.4
20	3	3	102.14	89.3	59.66
20	4	3	100.83	77	60.45
70	1	6	140	182.74	66.82
70	3	3	157	180.45	56.15
70	4	3	110.9	99.76	67.35

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