

Pre-column derivatization of amino acids from nigella sativa L seed hydrolysates by reversed phase HPLC

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Date of acceptance 4/10/2007

Abstract:

A rapid and sensitive method for analysis of amino acid hydrolysates of nigella sativa L seed has been developed using O-phthalaldehyde (OPA) as a pre-column derivatizing agent. OPA reagents in the presence of mercaptoethanol react rapidly with primary amino acids (less than 60 sec.) to form isindole derivatives which easily separated with good selectivity on ODS column.

Resolution of amino acid derivatives is carried out with a methanol gradient in 0.01 M aqueous sodium acetate, pH 7.1.

The quantitation of amino acid derivatives is reproducible within an average relative deviation of + 1.4%. The linearity for most amino acids were more than 0.9993 with detection limit of 0.2 ppm. 15 amino acids were detected in the analysis of the seed protein hydrolysate. The presence of glutamic acid, alanine, leucine, cystine, phenylalanine, aspartic acid in large quantities. The common separated amino acids were detected by U.V at 338 nm within 21 minutes.

Introduction:

Protein contents and amino acid level in blood serum have a wide useful nutritional effect. The amino acid levels in serum, has significant relation to growth rate. Total serum lipids, triglycerides, albumin and globulin, the activity of serum phosphatase and transaminases [1]. The amino acid supplementation from different sources has significantly different nutritional effects and pharmacological activities [2, 3]. Nigella sativa L seeds are used in different countries for many purposes.

It is used as diuretic, carminative and flavoring agent in Egypt, and for cheese flavoring and baking products in Syrians and Armenians, it is oil used for medical purposes such as for treatment of asthma, respiratory oppression, headaches, diuretic and other diseases [4].

Antibacterial and antifungal properties of the oil were reported by EL-Dakhakhny [5, 6].

However, Rethi et al. [7], Akram Khan [8] and Salomi [9] studies the chemical composition related to nutritive value and pharmacological activities of nigella oil seed.

Classical ion-exchange chromatography with subsequent post-column ninhydrin derivatization still remains as reliable method for routine amino acid analysis, but the classical ninhydrin reagent has fundamental limitations, characterized by slow reaction rate, high reaction temperature and long analysis times, the predominance of ninhydrin is now challenged by more sensitive, faster

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analysis and more reproducible method using O-phthalal-dehyde (OPA) on reversed phase column using high-performance liquid chromatography HPLC[10,11].

The aim of this work firstly is to investigate the amino acids constituent of IRAQI nigella sativa L seed, in order to evaluate the importance of relative wide usage of seeds in IRAQ. Secondly to adapted a rapid and sensitive quantitative method for determination of amino acids from plant extracts, which has many applications in the area of protein chemistry and clinical chemistry.

Clinical significance:

Amino acids are organic compounds containing both an amino group (NH₂) and a carboxyl group (COOH). Amino acids are the principal constituents of protein molecules, either they are linked together by peptide bonds into long chains containing from 50 to many thousands of amino acids. Some 40 different amino acids have been isolated from various proteins. Essential amino acids must be supplied in the diet. Those considered essential for vertebrates are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine[8].

Congenital defects in amino acid metabolism are responsible for a variety of pathologic conditions, such as phenylketonuria, tyrosinosis, histidinemia, maple syrup urine disease and cystinuria. Some of these result in mental retardation so that early detection and treatment is important. The amino acid concentration in plasma is relatively stable, with transient elevations occurring after high protein meals. A slight elevation may be found in persons with diabetes mellitus or impaired renal function. Significantly, elevated levels are seen only in severe liver disease, especially massive hepatic necrosis caused by toxic agents [12, 13].

The urinary output of amino acids is of great clinical significance. Increased levels of specific amino acids may indicate the presence of inborn errors of amino acid metabolism. More commonly, aminoaciduria indicates some underlying generalized disease, such as liver failure, metal poisoning, acute tubular necrosis, severe wasting or congenital disease. The finding of an elevation of glycine in cerebrospinal fluid is diagnostic of nonketotic hyperglycinemia [9].

Other disorders where amino acid analysis may have clinical importance: arteriosclerosis, arthritis, endocrine disorders, hemoglobin disorders, infections (bacterial, viral), liver diseases, muscle diseases, neoplastic diseases, neurological disorders (amyotrophic lateral sclerosis, Parkinson's disease, schizophrenia), nutritional disturbances (parental feeding control, obesity, starvation, vitamin deficiencies, renal failure) and stress (burn, injuries [multiple or to the head], postoperative [14, 15].

Experimental:

Oil:

The nigella sativa L seed were obtained from the local market, which is typical of commercially available products, cleaned and ground. Oil was obtained by soaked seed in hexane and separating the oil using rotary evaporator. Ethanol extracted oil were obtained by soxhlet extraction procedure.

Material:

Methanol HPLC GRADE (Fluka). Solutions of amino acid standards 2.5 μ mole / ml, OPA reagent, 2-mercapto ethanol, sodium acetate, were obtained from (Aldrich chem... Co. Ltd). The OPA reagent, were prepared by dissolving 50 mg of OPA in 1.25 ml of absolute methanol followed by addition of 50 ml of 2-mercapto, ethanol and 11.2 ml of 0.4 ml

sodium borate PH (9.5). This solution stable for two weeks. (11)

Chromatographic System:

The HPIC system consist of two Shimazu model LC-6A pumps (Koyoto japan), SIL-6A automated system controller for generation of elution gradients and a Shimazu SPD-6AV UV-visible detector equipped with 8 μ flow cell. The sample introduces into the column through Rheodye 7125-sample injector with 20 μ injection loop. The data were processing and analyzed by RC-4A data processors.

The column used was Shimpack-ODS (250 \times 4.6 mm 1.d), 5 μ m particle size. Gradient were formed between two degassed solvents. Solvent A 5% methanol in 0.1 N sodium acetate buffer (PH7.0), B methanol, further detail of chromatographic procedure are given in figure legends.

Derivatization Procedure:

The general procedure for derivatization was as follow, 10 μ L of aliquots of standard or standard or unknown sample were mixed with 10 μ L of OPA reagent after 1 minute, and 50 μ L of 0.1 M sodium acetate (pH7.0) were added. The solution mixed and 20 μ L sample was subjected to analysis.

Results and Discussion:

Table 1 shows high – speed separation of amino acid hydrolysis from nigella seeds extract under standard condition summarized in experimental section. The analysis time is 21 minutes. Typical cycle time between injections is 30 min (with 7 minutes of column reequilibrium at 1.5 ml/min).

The retention time of amino acid or reversed phase column depend on the structure and pka of each amino acid as shown in table (2).

The reagent composed of ortho – phthalaldehyde((OPA) was a selective reagent react with primary amino acids (10). The amino acid mixture derivatized with OPA can be successfully resolved by HPLC using gradient elution with methanol and 5- μ m particle size ODS column.

Methanol has found a wide use in separation of amino acids, peptides and proteins. A mainstay separation is that of derivatized amino acids. Engelhardt et al. [16, 17] reviewed the classical pre – column derivatization methods, including phenylisothiocyanate (PITC) to give PTH (Phenyl Thio Hydantion) derivative, dimethyl amino naphthalene sulfonyl (dansyl) chloride, o-phthalaldehyde (OPA) and 9-fluoromethyl chloroformate (FMOC). In this study the good resolution of 15 OPA-amino acids using gradient elution program on ODS column were obtained as in table 1. A major advantage to OPA is that the reactions quite clean no side reactions typically occur with low detection limits.

The amino acid composition of nigella sativa L. Seeds are summarized in table -1 . The highest percentage were by glutamic acid followed by threonine while the lowest percentage by glutamine followed by cystine our results have good agreement with Babayan et al. [2] who found that the highest values were by glutamic acid. Seven essential amino acids were detected through seed analysis, i.e. leucine, isoleucine, valine, methionine, phenylalanine threonine and tyrosine which from about 50% from the total amino acids. While the non-essential amino acid represented 50%, so the seeds protein of nigella sativa is rich in glutamic acid threonine, aspartic acid, leucine, Isoleucine, glycine tyrosine and cystine. Each amino acid has a vital role in biochemical activity and nutrient value i.e. Tyrosine, it is speculated that tyrosine availability would lead to control stimulator by norepinephrine (NE) inhibitory neurons in

brain stem [18], so tyrosine is proposed to increase the synthesis and turn over rate of NE [18, 19], some study [18] indicate, that a deficient protein diet 7% result a higher NE turn over rate in heat and higher urinary NE excretion.

However, cystine with naturally present vitamin E in nigella sativa oil reduce the toxicity of cis – platin in rat [19] by high tendency to protect from cisplatin induce falls in leucocytes counts, haemoglobin level and mean osmotic fragility of erthrocytes and also prevented the increase in haematocrit.

The study, suggest that cystine, natural antioxidant and 0.1 M of nigella sativa may be a promising compound for reducing cis –platin (chemotherapy)toxic side effects, including nephrotoxicity.

Conclusion:

This study demonstrates the analysis of the standard hydrolysate amino acids in 21-30 min with good resolution, column stability and sensitivity. Using precolumn derivatization – with (OPA). The detection limit 0.2 ppm .

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Table 1 : Quantitation of amino acids in nigella sativa seed. (As a percent of total amino acids).

No.	Amino acids	Rt min	Percentage
1	Glutamic acid (Glu)	2.30	19.20
2	Aspartic acid (Asp)	2.89	7.81
3	Serine (Ser)	3.33	3.5
4	Asparagine (Asn)	4.91	
5	Glutamin (Gln)	5.99	1.0
6	Histamin (His)	6.44	2.5
7	Cystine (Cys)	8.93	5.3
8	Glycine(Gly)	12.01	6.0
9	Treonine (Thr)	12.63	18.2
10	Alanine (Ala)	13.97	5
11	Tyrosine (Tyr)	15.4	5.0
12	Methionine (Met)	15.76	3.0
13	Valin (Val)	17.52	3.5
14	Tryptohan (Tyr)	18.15	
15	Phenylalanine (Phe)	19.036	6.7
16	Leucine (Leu)	19.36	6.8
17	Isdeucine (Ile)	20.69	6.5
18	Iysine (Iys)	21.19	

Table 2 amino acid abbreviations and pKa values.

Name	Abbreviations			PKa values	
	3letter	1 letter	- COOH	-NH ₃	R group
Alanine	Ala	A	2.34	9.69	
Arginine	Arg	R	2.17	9.04	12.48
Asparagine	Asn	N	2.01	8.8	
Aspartic acid	Asp	D	1.89	9.60	3.65
Cysteine	Cys	C	1.96	8.18	10.29
Glutamine	Gln	Q	2.17	9.13	
Glutamic	Glu	E	2.19	9.67	4.25
Glycine	Gly	G	2.34	9.6	
Histidine	His	H	1.8	9.17	6.00
Isoleucine	Ile	I	2.35	9.68	
Leucine	Leu	L	2.36	9.60	
Lysine	Lys	K	2.18	8.95	10.52
Methionine	Met	M	2.28	9.2	
Phenylalanine	Phe	F	1.83	9.12	
Serine	Ser	S	2.21	9.15	
Threonine	THr	T	2.11	9.62	13.6
Tryptophan	Trp	W	2.38	9.39	
Tyrosine	Tyr	Y	2.2	9.11	10.06
Valine	Val	V	2.32	9.61	

مشتقة ما قبل العمود سائل كروماتوگرافي عالي الأداء لفصل الاحماض الامينية في بذور الحبة السوداء

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الخلاصة

تم تثبيت طريقة سريعة وحساسة لتحليل الأحماض الامينية لزيت بذور الحبة السوداء باستخدام المشتقة ما قبل العمود لمادة اورثوفثالديهايد (OPA) وبوجود المركب ايثانول يتم التفاعل مع الاحماض الامينية الاولية بوقت قصير لا يتعدى 1 دقيقة مكونا مشتقة الايزواندول مع الاحماض الامينية. تم حصول الفصل التام لمشتقات الاحماض الامينية على عمود الطور العكوس باستخدام طريقة الاسترجاع التدريجي، باستخدام الميثانول والماء اللايوني او منظم الحامضية نوع الصوديوم (pH 7.1). التعيين الكمي لمشتقات الاحماض الامينية قد تم بوقت لا يزيد عن 25 دقيقة . بينت الدراسة وجود 15 حامض أميني في المستخلص الكحولي للحبة السوداء والتي لها فوائد صحية وطبية واسعة مع مكونات الزيت الاخرى .