Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1088 (2005) 158–168
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Source identification of Indian opium based on chromatographic fingerprinting of amino acids

M.M. Krishna Reddy[a], Priyankar Ghosh[a], S.N. Rasool[a], R.K. Sarin[a], R.B. Sashidhar[b,*]

[a] *Central Forensic Science Laboratory, Directorate of Forensic Science, Ministry of Home Affairs, Ramanthapur, Hyderabad 500013, India*
[b] *Department of Biochemistry, University College of Science, Osmania University, Hyderabad 500007, India*

Available online 8 December 2004

Abstract

Total and free pool of amino acids was determined in Indian opium samples using liquid chromatography (LC) with post-column *o*-pthalaldehyde derivatization followed by its fluorimetric detection. The limit of detection (LOD) was found to be in the range of 2–10 pmol with a signal to noise ratio of 3:1 and limit of quantitation (LOQ) was found to be in the range of 7–31 pmol with a signal to noise ratio of 10:1. The recovery of amino acids was found to be in the range of 86–103%. A total of 124 Indian opium samples were collected from the states of Madhya Pradesh (MP), Uttar Pradesh (UP) and Rajasthan (Raj), covering 14 licit opium growing divisions of India were chromatographically fingerprinted for the presence of various amino acids. The amino acids identified in sample hydrosylate included D, T, S, E, G, A, V, I, L, Y, F, H, K and R, while the analysis of free pool of amino acids (80% aqueous ethanol extract) indicated the presence of D, T, S, E, A, V, I, L, Y, H, K respectively. Multiple discriminant analysis was applied to the quantitative total amino acid data to determine an optimal classifier in order to evaluate the source of Indian opium. The foremost amino acid variables that accounted for the true discrimination were identified as D, E, G, A, F and K in evaluating the geographical origin of Indian opium and the predictive value based on the discriminant analysis was found to be 90% in relation to the source of opium samples. Chemometrics performed with amino acid analytical data was used successfully in discriminating the licit opium growing divisions of India into three major groups, viz. groups I, II and III. The methodology developed may find wide application in forensic analysis.

Keywords: Indian opium; Amino acids; Liquid chromatography; Discriminant analysis and source identification

1. Introduction

Opium is partly dried latex obtained from the unripe capsules of *Papaver somniferum* Lynn. It is cultivated mainly in the Indian subcontinent, Turkey, China, France, Spain, and Australia. India is the major licit producer and a principal supplier of opium to the world market for medicinal purposes. About 20,000 ha of land has been used in the specified areas of Madhya Pradesh (MP), Uttar Pradesh (UP) and Rajasthan (Raj) of India for the cultivation of opium in India under the supervision of Central Bureau of Narcotics, Government of India [1]. However, opium being a narcotic commodity, its illicit cultivation, production, manufacturing, possession, sales, purchase and trafficking attract stringent punishment under Narcotic Drugs and Psychotropic Substance (NDPS) act 1985, Government of India (rupees 2 lakhs, equivalent to US$ 4200 fine and 10–20 years rigorous imprisonment, which may go up to death penalty if the offence is repeated).

Opium ranks second most important item of globally seized commodity after cannabis (*Cannabis sativa* L.). The world-wide report brought out by the Office of National Drug Control Policy, USA indicates that there was an increase of 152% in the production of illicit opium during the period 1985–1992 [2]. According to United Nations Office on Drugs and Crime report (2003), the illicit opium production in the world was estimated to be 4000–6000 metric tones per annum [3]. Characterization of seized narcotic samples through laboratory analysis has long been recognized as a valuable tool to the law enforcement agencies in controlling the clan-

* Corresponding author. Fax: +91 40 27016868.
E-mail address: sashi_rao@yahoo.com (R.B. Sashidhar).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.10.109

destine opium production and its illicit trafficking. Such characterization not only provides sufficient proof of identity for judiciary, but may also assist in establishing the trafficking patterns and eventually the source of the illicit opium.

Attempts have been made to find out the source of opium by analyzing the various chemical constituents including free amino acids in opium to help the law enforcement agencies for checking its illicit production and trafficking under the United Nations opium characterization programme [4].

Amino acids were found to be important building blocks in the biosynthesis of alkaloids. The aromatic amino acids, phenylalanine and tyrosine have been shown to be the precursors for the biosynthesis of principal opium alkaloids (morphine, codeine, thebaine, papavarine and narcotine) [5]. Since the relative content of opium alkaloids varies considerably depending upon the region where the poppy (*P. somniferum*Lynn) is grown, a similar variation may also be found in the amino acid composition. A number of investigators have demonstrated a relationship between the amino acid composition of various plants and their mineral nutrition [6]. The soil content of potassium, phosphorous, sulfur and a number of trace elements such as iron, copper, zinc, manganese and molybdenum markedly affects the relative content of free amino acids in plants. The mineral composition of the soil where the poppy is grown will probably vary from one geographical region to another, as is indicated by the variation in the mineral content of opium itself [7,8]. Certain other environmental factors, such as temperature, light and water supply during growth were also found to influence the amino acid composition of poppy plant [6].

Earlier, profiling of free pool of amino acids was done under the United Nations programme for opium characterization and its origin determination by using paper chromatography (qualitative study) [9] and spectrophotometrically using ninhydrin as derivatizing agent [10]. These reported methods have certain limitations like low sensitivity, highly laborious, time-consuming and no quantitative data is available in the contemporary literature on the analysis carried out by the traditional chromatographic method.

The adaptability of liquid chromatography (LC) with software control of gradients improved the amino acid analysis producing superior resolution with only two buffers. Use of *o*-pthalaldehyde instead of ninhydrin in the amino acid analysis increases the stability of the derivative and its sensitive detection to the picomole range (<5 pmol) by LC with fluorometric detection [11]. The use of *o*-pthalaldehyde as derivatizing reagent for the fluorometric detection of amino acids is continuously increasing since 1971 [12]. In the recent past, *o*-pthalaldehyde derivatization procedure is also a routinely used technique in analyzing the amino acids in various biological samples. During the period from 1992 to 1998, the use of *o*-pthalaldehyde as derivatizing agent was about 70% of all derivatizations performed in LC for amino acid analysis [13]. Though LC-based techniques have been utilized by the researchers for the determination of amino acids in various plant products [14,15], no LC-based method was adopted so far for the determination of amino acids in opium with forensic application.

In the present communication, we report quantitative analysis of total amino acids in 124 samples of licit opium collected from three states of India covering 14 divisions. The sample analysis was based on LC coupled with post-column derivatization system using *o*-pthalaldehyde as a fluorescent derivatizing agent [16]. Further, the analytical data of amino acid profile has been subjected to multivariate analysis to determine the common chemical indices (total amino acids and free pool of amino acids) to build supervised classifier (discriminant analysis) for the discrimination of the licit opium growing divisions of India in order to pin point its source.

2. Experimental

2.1. Materials and reagents

All solvents used were of liquid chromatography grade unless otherwise stated. Tri sodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), perchloric acid, boric acid, sodium hydroxide, sodium hypochlorite, ethyl alcohol, *o*-pthalaldehyde, sodium carbonate, potassium sulfate, hydrogen peroxide (30%, v/v), formic acid (88%, v/v), hydrobromic acid (48%, v/v) and Brij-35 were of analytical grade reagents and procured from E. Merck (Mumbai, India); *N*-acetyl-L-cysteine was procured from Fluka (Milwaukee, WI, USA) and *n*-caprylic acid was procured from Spectrochem Ltd. (Mumbai, India). Standard amino acids were procured from Sigma (St. Louis, MO, USA). The internal standard (DL-norleucine) was procured from Mann Research Laboratory (NY, USA). Ultra pure water from Milli-Q system (Millipore, Bedford, MA, USA) was used in all cases. Authentic samples of opium ($n = 124$) were collected in semi-dried state from the states of Madhya Pradesh, Uttar Pradesh and Rajasthan covering 14 divisions of licit opium growing divisions of India. Government Opium and Alkaloids Works situated at Ghazipur, UP and Neemuch, MP facilitated the sample collection. The number of samples collected from each licit opium growing divisions are, viz. Garoth (3 samples, MP), Jaora (3 samples, MP), Mandsaur (18 samples, MP), Neemuch (12 samples, MP), Ujjain (7 samples, MP), Bhilwara (8 samples, Rajasthan), Chittorgarh (15 samples, Rajasthan), Jhalwar (9 samples, Rajasthan), Kota (17 samples, Rajasthan), Pratapgarh (6 samples, Rajasthan), Barabanki (6 samples, UP), Bareilly (6 samples, UP), Faizabad (6 samples, UP) and Tilhar (8 samples, UP). Nineteen samples were selected for the free pool of amino acids by random sampling method. These include one sample each from Barabanki, Bareilly, Bhilwara, Chittorgarh, Faizabad, Garoth, Jaora, Jhalwar, Pratapgarh, Tilhar, Ujjain, two samples from Kota, three samples each from Mandsaur, Neemuch. Five samples were selected for the analysis of tryptophan, cystic acid and methionine sulfone. These include one sample each from Neemuch, Jaora, Mandsaur, Pratapgarh and Bareilly. The samples

obtained from the above sources were dried at 105 °C for 2 h, to a constant weight and powdered in a high-speed mechanical blender. Powdered samples were kept in a vacuum dessicator, under anhydrous calcium chloride until further use. The values reported in the present study are based on dry weight basis. All buffers were filtered through 0.45 μm membrane filter (Millipore, Bedford, MA, USA).

2.2. Mobile phases

Eluent A ($Na_3C_6H_5O_7 \cdot 2H_2O$, 0.067 M): The buffer was made by dissolving tri sodium citrate (19.6 g) in ultra pure water (500 ml), followed by addition of absolute ethanol (70 ml). Later, the pH was adjusted to 3.2 with 60% perchloric acid and finally the volume was made up to 1 l with ultra pure water using grade 'A' volumetric flask.

Eluent B {$Na_3C_6H_5O_7 \cdot 2H_2O$ (0.2 M) and boric acid (0.2 M)}: The buffer was made by dissolving the $Na_3C_6H_5O_7 \cdot 2H_2O$ (58.8 g), boric acid (12.4 g) in ultra pure water (500 ml). Later, the pH was adjusted to 10 with 4 M sodium hydroxide and finally the volume was made up to 1 l with ultra pure water using grade 'A' volumetric flask.

Sample buffer {$Na_3C_6H_5O_7 \cdot 2H_2O$ (0.067 M), pH 2.2}: The buffer was made by dissolving $Na_3C_6H_5O_7 \cdot 2H_2O$ (9.8 g) in ultra pure water (400 ml), followed by addition of perchloric acid (8 ml), *n*-caprylic acid (0.05 ml) and finally the volume was made up to 500 ml with ultra pure water using grade 'A' volumetric flask.

o-Pthalaldehyde reagent buffer (pH 10): Sodium carbonate (40.7 g), boric acid (13.6 g) and potassium sulfate (18.8 g) were dissolved in ultra pure water (500 ml). Finally, the volume was made up to 1 l with ultra pure water using a grade 'A' volumetric flask.

o-Pthalaldehyde derivatizing reagent: *N*-acetyl-L-cysteine (500 mg) was dissolved in *o*-pthalaldehyde reagent buffer (400 ml) and *o*-pthalaldehyde (400 mg) were dissolved in ethanol (7 ml). The derivatizing reagent was prepared by mixing the latter to former with stirring followed by addition of 10% Brij-35 (2 ml) to the above solution. Finally, the volume was made up to 500 ml with *o*-pthalaldehyde reagent buffer using grade 'A' volumetric flask.

Hypochlorite reagent for online oxidation was prepared by adding 0.4 ml of the commercial sodium hypochlorite solution (4% (w/v) of available chlorine) to 1 l of *o*-pthalaldehyde reagent buffer.

Performic acid: The performic acid was prepared by mixing 90 ml of formic acid (88%, v/v) and 10 ml of H_2O_2 (30%, v/v). This mixture was thoroughly agitated for 5 min. Later, the mixture was allowed to stand at ambient temperature for 1 h, and then cooled in ice bath for 15 min before use.

2.3. Preparation of standard solutions

2.3.1. Stock solution

Stock solution of standard mixture of amino acids were made by accurately weighting each amino acid in an analytical balance (Mettler, Greitensee, Switzerland), dissolving the same into sample buffer and finally the volume was made to 10 ml using grade 'A' volumetric flask in order to obtain a final concentration of 10 μmol/ml. Stock solution contained the amino acids like cystic acid, aspartic acid, threonine, serine, glumatic acid, proline, glycine, alanine, valine, methionine sulfone, isoleucine, leucine, phenylalanine, tyrosine, histidine, lysine and argenine. Some standard materials were supplied as hydrochlorides. Corrections were made to express final concentration in nanomoles of each amino acid in its pure form. The calibration concentrations of reference standards used for the analysis were given in Table 2.

2.3.2. Internal standard solution

The internal standard solution was made by accurately weighing the norleucine in an analytical balance (Mettler, Greitensee, Switzerland), dissolving the same in to sample buffer and finally the volume was made to 5 ml using grade 'A' volumetric flask in order to obtain a final concentration of 10 μmol/ml. One nanomole per μl of internal standard (norleucine) was added to each calibration concentration of standard amino acid and in opium samples.

2.4. Sample hydrolysis

The opium samples were defatted by soxhlet extraction before the sample hydrolysis [14]. Samples were hydrolyzed by a reported method [17]. Acid (6 M HCl) hydrolysis was used for all amino acids except tryptophan, cysteine and methionine. Cysteine and methionine were first converted to cystic acid and methionine sulfone by performic acid oxidation followed by acid hydrolysis. Base hydrolysis (6 M NaOH) method was used for tryptophan only.

(a) Acid hydrolysis: Opium sample (100 mg) was accurately weighed in an analytical balance (Mettler, Greitensee, Switzerland) and transferred to a digestion tube; then 5 ml of 6 M HCl was added to the digestion tube and sealed under N_2 atmosphere. The sealed tube was placed in a hot air oven for 22 h at 110 °C.

(b) Performic acid oxidation and acid hydrolysis: Chilled performic acid (2 ml) was pipetted into a digestion tube containing 100 mg of opium sample. The digestion tube was then placed in an ice bath (0 °C) for 16 h. A 0.3 ml of hydrobromic acid (48%, v/v) was added to the tube, and the same was allowed to stand in an ice bath for 15 min. The contents of the tube were dried on a rotary vacuum evaporator at ≤60 °C. Subsequently the samples were hydrolyzed using 6 M HCl.

(c) Base hydrolysis: Opium sample (100 mg) was accurately weighed in an analytical balance (Mettler, Greitensee, Switzerland) and transferred to a digestion tube; then 5 ml of 6 M NaOH was added to the digestion tube and sealed under N_2 atmosphere. The sealed tube was placed in a hot air oven for 22 h at 110 °C.

(d) Sample preparation after hydrolysis: The hydrolyzed samples were cooled to room temperature, filtered and made up to 25 ml in a grade 'A' volumetric flask. An aliquot (5 ml) of sample hydrolysate was transferred into a round bottom flask and evaporated to dryness on a rotary evaporator set at ≤60 °C and later redissolved in 2 ml of sample buffer. After thorough mixing on a vortex, the reconstituted hydrolysate was filtered through a Millipore filter membrane (0.45 μm). An aliquot of 3 μl of the sample extract was injected into LC system with the aid of an auto sampler.

2.5. Extraction of free amino acids

The free pool of amino acids present in the opium samples was extracted separately by using 80% ethanol. The method adopted for extraction is as follows.

Opium sample (200 mg) was accurately weighed and transferred to a centrifuge tube; then, 2 ml of 80% aqueous ethanol was added to the tube and kept overnight so as to facilitate complete extraction. The mixture was vortexed for 5 min and centrifuged for 10 min at 2000 rpm. The supernant was decanted, and then 2 ml of 80% aqueous ethanol was added to the residue, vortexed for 5 min and centrifuged for 10 min at 2000 rpm. Again supernant was decanted and the procedure was repeated twice on the residue. Supernants were pooled and evaporated to dryness by rotary vacuum evaporator and the resultant mass was redissolved in 2 ml of sample buffer. A 3 μl of the sample extract was injected into liquid chromatographic system with the aid of an auto sampler.

2.6. Spiking studies

The standard amino acids were spiked to opium sample at a concentration of 200 nmol of each per ml of opium extract in order to assess the recoveries of each amino acid (injection volume 3 μl).

2.7. Chromatography

A Shimadzu LC-10AT LC system (Tokyo, Japan) equipped with a quaternary solvent delivery controller was used for delivering the mobile phase, and a PRR–2A peristaltic pump (Shimadzu, Tokyo, Japan) was utilized for delivering the post-column reagents. The mobile phase for amino acid analysis was delivered at a constant flow rate of 0.3 ml/min. The sodium hypochlorite and *o*-pthalaldehyde/*N*-acetyl-L-cysteine reagents were delivered at a constant flow rate of 0.3 ml/min in the above case. Samples were injected with a Shimadzu SIL-10AT auto sampler. The separation column and reaction coils were placed in Shimadzu CTO-10AT column oven. The oven temperature was maintained at 55 °C. The fluorescence intensity of the effluent was measured at the excitation and emission maxima of 348 and 450 nm, respectively, in a Shimadzu RF-10A$_{XL}$ spectrofluoromonitor, equipped with a xenon discharge lamp. A sodium type ion exchange (Shim pack ISC-07/S1504, Shimadzu, Tokyo, Japan) LC column was used for the separation of amino acids. The packing consisted of strongly acidic cation exchange resin of styrene-divinylbenzene packed in a stainless steel tube (150 mm × 4 mm i.d.). Guard column consisted of strong cation exchange resin having a sulfonic acid group was placed before the analytical column in order to protect the analytical column from clogging (ISC-07 Na, 50 mm × 4 mm i.d). Trap column (Shim pack ISC-30/S0504 Na, 50 mm × 4 mm i.d, Shimadzu, Tokyo, Japan) was placed in between the pump and auto sampler in order to trap ammonia.

Statgraphics (version 5.0, Statistical Graphics Corporation, MD, USA) and Microcal origin software's (version 6.0, Microcal Software Incorporation, Northampton, USA) were used for multivariate (discriminant) analysis and plotting the discriminant scores.

3. Results and discussion

Earlier, Fujiwara et al. [16] developed a post-column fluorimetric detection system for liquid chromatographic analysis of amino and imino acids qualitatively using *o*-pthalaldehyde/*N*-acetyl-L-cysteine reagent, which gives sensitive detection of secondary amino acids such as proline and was easier to handle than the thiols used in conventional *o*-pthalaldehyde/thiol reagent. Further, *N*-acetyl-L-cysteine reagent used as a reducing agent was odorless and stable. *o*-Pthalaldehyde post-column derivatization was easily achieved in the present investigation with a maximum fluorescence yield at a temperature of 55 °C in 0.1–1 min [18].

A gradient programme was used for the separation of amino acids in aqueous ethanol extract and in acid hydrosylate samples. The effect of mobile phase composition on peak resolution of amino acids was studied by varying the concentration of each eluent (buffer) with time. A suitable gradient programme was developed for the separation of amino acids (Table 1). Fig. 1 shows the profile of amino acids in opium sample after: (i) acid hydrolysis; and (ii) performic acid oxidation followed by acid hydrolysis. The chromatogram indicates good baseline separation with sufficient peak shapes of resolved amino acids. Fig. 2 shows the free pool of amino acids in the opium sample after 80% aqueous ethanol extrac-

Table 1
Gradient system used for the separation of *o*-pthalaldehyde derivatized amino acids

Time (min)	A (%)	B (%)
0	100	0
15.00	100	0
35.00	90	10
40.00	90	10
40.01	40	60
50.00	40	60
65.00	0	100
70.00	100	0

A: Eluent A (0.067 M tri sodium citrate, pH 3.2); B: Eluent B (0.2 M tri sodium citrate and 0.2 M boric acid, pH 10).

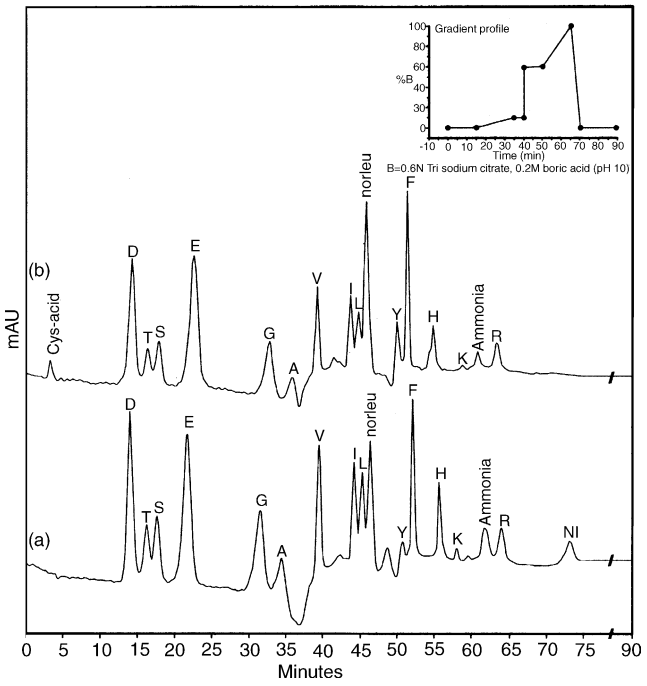

Fig. 1. Chromatographic profile of amino acids after: (a) acid hydrolysis; and (b) performic acid oxidation and followed by acid hydrolysis. D: Aspartic acid; T: threonine; S: serine; E: glutamic acid; G: glycine; A: alanine; V: valine; I: isoleucine; L: leucine; Y: tyrosine; F: phenylalanine; H: histidine; K: lysine; and R: arginine.

tion. Peak identification was done by comparing the retention times of amino acids in opium with that of the standard amino acids and also confirmed by spiking each individual reference standard amino acid to that of the opium sample. A total of 14 amino acids were detected in all the opium samples analyzed. The amino acids detected in sample hydrosylate were aspartic acid (D), threonine (T), serine (S), glutamic acid (E), glycine (G), alanine (A), valine (V), isoleucine (I), leucine (L), tyrosine (Y), phenylalanine (F), histidine (H), lysine (K) and argenine (R). Analysis of 80% aqueous ethanol extract indicated the presence of 11 free pool of amino acids. These were identified as D, T, S, E, A, V, I, L, Y, H, K, respectively. The amino acids like G, F and R were not present in free pool of amino acids. Two unidentified peaks in 80% aqueous ethanol extract were observed which were present in relatively higher amounts. However, the molecular identity of these unidentified peaks could not be ascertained. Proline (P) was not detected in sample hydrosylate as well as in 80% aqueous ethanol extract. The amino acids G, F, and R were not detected in free pool but were present in total amino acids. Interestingly, not all amino acids in free pool listed above were detected in every sample. The aspartic acid (D) in free pool was absent in Barabanki, Bareilly Chittorgarh and Jhalwar. Threonine (T) in free pool was not detected in Garoth. Tyrosine (Y) in free pool was not detected in Bareilly, Bhilwara, Garoth and Jhalwar. Lysine (K) in free pool was not detected in Bhilwara. Five samples were analyzed for tryptophan (W), cystic acid and methionine sulfone. Cystic acid was detected in all five-opium samples, but tryptophan and methionine sulfone were not detected in all opium samples analyzed. The limit of detection (LOD) was based on the concentration of the respective amino acids that gave a signal three times higher than the baseline noise (3:1). The LOD of amino acids were found to be in the range of 2.3–10.4 pmol per μl of injection (Table 2). The limit of quantitation of amino acids was found to be in the range of 7.16–31.44 pmol per μl injection (Table 2) with a signal to noise ratio of 10:1.

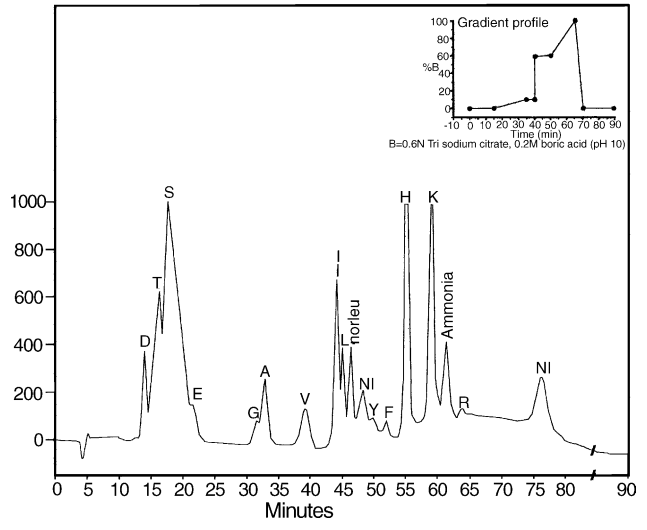

Fig. 2. Chromatographic profile of free amino acids after 80% aqueous ethanol extraction. D: Aspartic acid; T: threonine; S: serine; E: glutamic acid; G: glycine; A: alanine; V: valine; I: isoleucine; L: leucine; Y: tyrosine; F: phenylalanine; H: histidine; K: lysine; and R: arginine.

Each amino acid was detected, quantified on the basis of internal standard (norleucine) method. The calibration plot was based on linear regression analysis and it was found to be linear with a correlation coefficient (r^2) value greater than 0.995 (Table 2). Table 3 shows the percent composition (g%) of amino acids in opium samples analyzed. The total amino acid content was found to vary over a wide range from 0.6 to 21 g% with majority of the samples falling in the range of 2.5–6.5 g%. There was also a considerable variation in the relative composition of amino acids in different opium samples. The relative standard deviation (R.S.D.) of the amino acid content in all opium samples was found to be in the range of 0.36–4.59%. Table 4 shows the percent composition of free pool of amino acids detected in 19 opium samples. Table 5 shows the percent composition of cystic acid in five opium samples. The R.S.D. for retention time and peak area of the amino acids detected in opium samples after acid hydrolysis and the after the extraction with 80% aqueous ethanol are depicted in Table 7. The R.S.D. of retention time and peak area for individual amino acids were found to be less than 1.2 and 6.5% for inter day analysis and less than 1 and 5% for intra day analysis, respectively, suggesting excellent reproducibility. Known amount of individual standard amino acids (200 nmol/ml) were added to the sample hydrosylate in order assess the recovery of amino acids. Recoveries of amino acids were found to be in the range of 86–103% (Table 2).

Table 2
Linearity, correlation, LOD, LOQ and recovery of amino acids in opium

Serial no.	Amino acid	Calibration Concentrations (nmol)	Linear regression equation ($y = mx + c$)	Correlation coefficient (r^2)	Limit of detection (LOD, pmol)	Limit of quantitation (LOQ, pmol)	Recovery (%)
1	Cystic acid	1–3	$24.3267x - 0.21055$	0.9994	5.25	11.76	95
2	Aspartic acid	1.5–3.5	$17.1325x - 0.2318$	0.9987	5.80	17.60	94
3	Threonine	2–4	$25.1621x - 0.099$	0.9970	8.81	26.7	89
4	Serine	2–4	$18.9685x - 0.095$	0.9972	7.68	23.27	91
5	Glutamic acid	1.5–3.5	$17.7281x - 0.3184$	0.9999	8.36	25.34	101
6	Glycine	2.5–6.5	$7.14213x - 0.2933$	0.9983	6.71	20.4	97
7	Alanine	2–6	$14.1911x - 0.2342$	0.9998	10.4	31.44	99
8	Valine	2–4	$12.6193x - 0.2020$	0.9998	3.31	10.05	97
9	Isoleucine	2–4	$22.074x - 0.1165$	0.9950	4.96	15	100
10	Leucine	2–4	$36.1492x - 0.0424$	0.9998	5.56	16.9	103
11	Tyrosine	1–3	$35.9388x - 0.1730$	0.9960	8.83	26.8	97
12	Phenylalanine	1–3	$24.643x + 0.2105$	0.9997	7.4	22.54	100
13	Histidine	1–3	$21.1544x - 0.1751$	0.9999	2.3	7.16	99
14	Lysine	1–3	$87.5753x - 0.040$	0.9994	9.1	27.57	86
15	Arginine	1–3	$32.901x - 0.2674$	0.9992	7.07	21.41	99

3.1. Chemometrics

Chemometric techniques are of great significance in the study of complex biological matrices including opium in relation to their characterization and classification based on the biochemical variables [19]. Among multivariate methods, discriminant analysis is considered to be an important classical parametric tool for grouping samples wherein the origin of the samples is known. The main purpose of this statistical model is to estimate an adequate linear function so as to predict and classify the samples of unknown origin to one group or to the other, successively. The discriminant analysis looks for one or more functions of quantitative measurements that will help to discriminate among multivariate observations, which can be classified into groups [20]. Multiple linear discriminant analysis was applied to the quantitative total amino acid data in the present study to provide a mathematical tool for finding the optimal classifier based on total amino acid profile in order to minimize the classification errors.

Software-based multiple discriminant analysis (MDA) programme was used as a feature selection approach that provides better classification. Pictorial representation of multivariate data into a two-dimensional projection with minimum classification error was obtained by plotting the discriminant scores. Discriminant functions were derived and plotted as a graph (Fig. 3). From the Fig. 3, it is evident that the samples from Kota (14/17) were classified as one group (group I). The samples from Garoth (3/3), Mandsaur (17/18), Neemuch (11/12), Ujjain (7/7), Pratapgarh (5/6) and Jaora (2/3) were classified as a separate group (group II). Samples from Barabanki (3/6), Bareilly (6/6), Bhilwara (6/8), Chittorgarh (13/15), Faizabad (5/6), Jhalwar (8/9), Tilhar (8/8) were classified as one group (group III). One sample each from the divisions of Jaora, Kota and Neemuch were misclassified into group III. Two samples from Jhalwar, Bhilwara and one sample from Chittorgarh were misclassified into group I. Very few samples (8/124) were found to be outside the classified groups (Table 6). Majority of the samples originating from the divisions of Garoth, Jaora, Mandsaur, Neemuch, and Ujjain belong to state of Madhya Pradesh were classified into group II. Interestingly, the Pratapgrah (Rajasthan state) division is geographically situated very close to the border of Madhya Pradesh state was also classified in to group II. Majority of the samples originating from Barabanki, Bareilly, Bhilwara, Chittorgarh, Faizabad, Jhalwar, and Tilhar, which were, segregated in to group III, which includes opium samples originating from the states of Uttar Pradesh and Rajasthan. Based on the discriminant analysis, the predictive value in relation to the source of opium samples was found to be 85%. Further, the variable reduction process was adopted in order to pin point the amino acid variables that can give

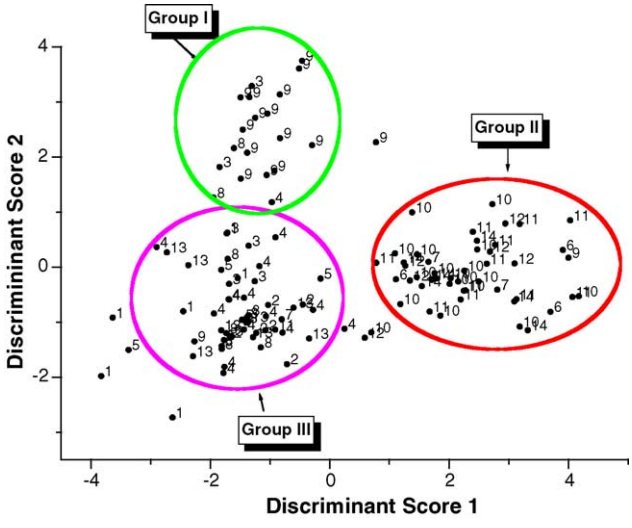

Fig. 3. Multivariate projection of the concentrations of 14 amino acids in the plane of two greatest discriminants for opium samples collected from licit opium growing areas of India. (1) Barabanki; (2) Bareilly; (3) Bhilwara; (4) Chittorgarh; (5) Faizabad; (6) Garoth; (7) Jaora; (8) Jhalwar; (9) Kota; (10) Mandsaur; (11) Neemuch; (12) Pratapgarh; (13) Tilhar; and (14) Ujjain.

Table 3
Percent composition (g%) of total amino acids in Indian opium[a]

Sample	Division[b]	Group	D	T	S	E	G	A	V	I	L	Y	F	H	K	R	Total
1[c]	Barabanki	1	0.145	0.067	0.052	0.301	0.310	0.877	0.079	0.080	0.179	0.020	0.094	0.040	0.176	0.028	2.448
2	Barabanki	1	0.189	0.125	0.087	0.463	0.570	2.238	0.162	0.150	0.243	0.016	0.167	0.080	0.189	0.047	4.726
3	Barabanki	1	0.168	0.101	0.080	0.398	0.390	0.178	0.143	0.140	0.161	0.033	0.187	0.060	0.129	0.045	2.213
4	Barabanki	1	0.139	0.098	0.071	0.525	0.310	0.108	0.126	0.100	0.116	0.087	0.162	0.060	0.478	0.050	2.43
5	Barabanki	1	0.171	0.108	0.084	0.607	0.400	2.289	0.157	0.130	0.154	0.059	0.187	0.070	0.210	0.063	4.689
6	Barabanki	1	0.456	0.125	0.066	0.806	0.151	2.320	0.139	0.110	0.206	0.032	0.190	0.053	0.240	0.072	4.966
7	Bareilly	2	0.348	0.097	0.072	0.650	0.100	0.212	0.145	0.120	0.167	0.022	0.239	ND	0.167	0.068	2.407
8	Bareilly	2	0.123	0.035	0.031	0.198	0.040	0.086	0.057	0.040	0.059	0.038	0.121	0.020	0.093	0.023	0.964
9	Bareilly	2	0.096	0.030	0.025	0.172	0.030	0.074	0.043	0.040	0.560	ND	0.124	0.020	0.053	0.031	1.305
10	Bareilly	2	0.242	0.081	0.065	0.500	0.070	0.231	0.110	0.090	0.137	0.069	0.234	0.050	0.207	0.033	2.119
11	Bareilly	2	0.383	0.101	0.080	0.726	0.100	0.216	0.150	0.130	0.186	0.073	0.318	0.070	0.252	0.067	2.852
12[c]	Bareilly	2	0.230	0.064	0.044	0.472	0.060	0.233	0.106	0.080	0.116	0.016	0.183	0.050	0.149	0.041	1.844
13	Bhilwara	3	0.583	0.137	0.117	0.983	0.842	0.935	0.011	0.220	0.245	0.182	0.503	0.100	0.410	0.090	5.358
14[c]	Bhilwara	3	0.406	0.108	0.094	0.763	0.575	0.665	0.013	0.160	0.242	0.138	0.276	0.070	0.166	0.071	3.747
15	Bhilwara	3	0.279	0.102	0.085	0.347	1.180	0.641	0.145	0.106	0.134	0.144	0.096	0.029	0.341	0.074	3.7
16	Bhilwara	3	0.141	0.043	0.046	0.275	0.044	0.094	0.068	0.060	0.075	0.049	0.127	0.030	0.093	0.034	1.179
17	Bhilwara	3	0.268	0.061	0.044	0.385	0.335	0.352	0.147	0.110	0.107	0.047	0.242	0.060	0.187	0.041	2.386
18	Bhilwara	3	0.377	0.107	0.111	0.726	0.104	0.229	0.160	0.190	0.101	0.332	0.069	0.260	ND	0.066	2.832
19	Bhilwara	3	0.236	0.054	0.030	0.430	0.336	0.313	0.130	0.080	0.094	0.061	0.205	0.050	0.043	0.043	2.105
20	Bhilwara	3	0.173	0.038	0.028	0.297	0.239	0.269	0.098	0.070	0.080	0.040	0.163	0.040	0.184	0.025	1.744
21	Chittorgarh	4	0.688	0.222	0.134	1.590	0.200	0.239	0.253	0.270	0.360	0.213	0.190	0.140	0.193	0.788	5.48
22	Chittorgarh	4	1.267	0.402	0.252	3.411	0.367	0.486	0.400	0.530	0.644	0.472	0.354	0.280	0.711	1.791	11.367
23	Chittorgarh	4	0.301	0.105	0.108	0.704	0.082	0.207	0.168	0.140	0.238	0.094	0.197	0.060	0.114	0.058	2.576
24	Chittorgarh	4	0.410	0.135	0.141	0.807	0.120	0.313	0.191	0.180	0.223	0.130	0.398	0.090	0.332	0.085	3.555
25	Chittorgarh	4	0.440	0.130	0.064	1.012	0.118	0.158	0.144	0.160	0.217	0.134	0.121	0.080	0.097	0.530	3.405
26	Chittorgarh	4	0.423	0.136	0.191	1.681	0.123	0.114	0.104	0.140	0.244	0.118	0.140	0.110	0.815	1.013	5.352
27	Chittorgarh	4	0.344	0.088	0.050	0.782	0.084	0.102	0.085	0.110	0.146	0.072	0.060	0.060	0.063	0.452	2.498
28	Chittorgarh	4	0.425	0.105	0.230	1.436	0.111	0.108	0.040	0.210	0.063	0.061	0.456	0.080	0.080	0.553	3.958
29	Chittorgarh	4	0.522	0.190	0.115	0.091	0.152	0.388	0.234	0.330	0.180	0.190	0.985	0.110	0.147	0.912	4.546
30[c]	Chittorgarh	4	0.353	0.166	0.053	1.067	0.110	0.328	0.181	0.250	0.210	0.130	0.560	0.100	0.155	1.049	4.712
31	Chittorgarh	4	0.342	0.130	0.060	0.901	0.105	0.208	0.130	0.220	0.123	0.128	0.494	0.080	0.122	0.571	3.614
32	Chittorgarh	4	0.409	0.138	0.239	1.897	0.114	0.126	0.126	0.210	0.163	0.125	0.430	0.180	0.071	0.532	4.76
33	Chittorgarh	4	0.295	0.107	0.113	0.646	0.089	0.353	0.158	0.150	0.180	0.089	0.352	0.070	0.247	0.053	2.902
34	Chittorgarh	4	0.503	0.151	0.151	0.905	0.402	0.430	0.196	0.018	0.245	0.472	0.121	0.042	0.700	0.081	4.417
35	Chittorgarh	4	1.900	0.764	0.418	6.410	0.598	1.390	0.990	1.020	1.220	0.935	0.676	0.480	0.581	3.866	21.246
36[c,d]	Faizabad	5	0.433	0.153	0.126	0.549	0.180	0.278	0.202	0.170	0.347	0.027	0.237	0.090	0.155	0.050	2.997
37	Faizabad	5	0.217	0.068	0.058	0.405	0.070	0.129	0.077	0.070	0.128	0.026	0.088	0.040	0.089	0.034	1.499
38	Faizabad	5	0.071	0.033	0.025	0.079	0.030	0.066	0.038	0.030	0.109	ND	0.038	0.030	0.019	0.020	0.597
39	Faizabad	5	0.223	0.079	0.065	0.305	0.300	0.449	0.097	0.080	0.147	0.013	0.102	0.040	0.152	0.040	2.092
40	Faizabad	5	0.131	0.096	0.056	0.382	0.390	1.749	0.115	0.110	0.138	0.033	0.142	0.060	0.231	0.035	3.668
41	Faizabad	5	0.166	0.058	0.050	0.364	0.060	0.273	0.089	0.080	0.098	0.078	0.130	0.040	0.100	0.030	1.616
42[c]	Garoth	6	0.540	0.156	0.111	0.982	0.170	0.176	0.235	0.200	0.290	0.121	0.636	0.100	0.538	0.085	4.34
43	Garoth	6	1.112	0.314	0.263	1.922	0.320	0.370	ND	0.980	0.698	0.033	1.422	0.260	1.329	0.170	9.193
44	Garoth	6	0.985	0.286	0.249	0.152	0.270	0.224	0.490	0.570	0.421	0.073	0.947	0.200	0.797	0.170	5.834
45	Jaora	7	0.661	0.206	0.180	1.089	0.210	0.178	0.293	0.280	0.364	0.118	0.712	0.110	0.597	0.115	5.113
46[c]	Jaora	7	0.328	0.088	0.089	0.437	0.090	0.175	0.118	0.120	0.158	0.069	0.270	0.082	0.318	0.056	2.398
47	Jaora	7	0.850	0.220	0.185	1.495	0.220	0.290	ND	0.570	0.510	0.493	1.359	0.200	1.054	0.128	7.574
48	Jhalwar	8	0.213	0.082	0.070	0.510	0.080	0.325	0.131	0.120	0.146	0.032	0.246	0.060	0.256	0.040	2.311
49	Jhalwar	8	0.101	0.058	0.038	0.224	0.040	0.201	0.070	0.050	0.111	0.025	0.062	0.040	0.058	ND	1.082
50	Jhalwar	8	0.308	0.076	0.073	0.547	0.570	0.816	0.264	0.100	0.216	ND	0.273	ND	0.154	0.170	3.569
51	Jhalwar	8	0.212	0.060	0.060	0.413	0.057	0.097	0.104	0.073	0.100	0.071	0.167	0.038	0.060	0.036	1.548
52	Jhalwar	8	0.208	0.042	0.030	0.263	0.263	0.189	0.117	0.060	ND	0.024	0.189	ND	0.163	0.027	1.575
53	Jhalwar	8	0.212	0.066	0.403	ND	0.132	0.341	0.059	0.010	0.114	0.090	0.183	ND	0.216	0.045	1.875
54	Jhalwar	8	0.195	0.054	0.059	0.354	0.060	0.117	0.104	0.080	0.103	0.053	0.185	0.030	0.063	0.031	1.488
55[c]	Jhalwar	8	0.140	0.059	0.053	0.330	0.059	0.146	0.091	0.070	0.094	0.059	0.138	0.030	0.057	0.042	1.368
56	Jhalwar	8	0.211	0.048	0.054	0.375	0.571	0.114	0.094	0.010	0.098	ND	0.182	0.030	0.182	0.038	2.012
57	Kota	9	0.419	0.113	0.109	0.954	0.661	0.740	0.341	0.204	0.274	0.094	0.310	0.084	0.268	0.061	4.632
58[c]	Kota	9	0.547	0.135	0.126	1.005	0.715	0.758	0.337	0.200	0.294	0.102	0.310	0.090	0.261	0.079	4.959

Table 3 (*Continued*)

Sample	Division[b]	Group	D	T	S	E	G	A	V	I	L	Y	F	H	K	R	Total
59	Kota	9	1.437	0.460	0.240	4.290	0.344	0.462	0.541	0.520	0.935	0.636	0.459	0.270	0.435	0.917	11.946
60	Kota	9	0.579	0.136	0.117	0.908	0.702	0.800	0.108	0.220	0.309	0.141	0.352	0.080	0.251	0.075	4.778
61	Kota	9	0.408	0.950	0.700	0.500	0.586	0.573	0.222	0.160	0.169	0.117	0.316	0.070	0.223	0.051	5.045
62	Kota	9	1.712	0.524	0.308	3.930	0.494	0.390	0.560	0.580	0.850	0.570	0.468	0.320	0.481	2.662	13.849
63	Kota	9	0.322	0.126	0.077	1.146	0.095	0.209	0.113	0.150	0.218	0.120	0.131	0.080	0.155	0.673	3.615
64	Kota	9	0.647	0.153	0.126	0.877	0.836	0.854	0.311	0.230	0.249	0.120	0.490	0.104	0.344	0.074	5.415
65	Kota	9	0.560	0.140	0.119	0.976	0.782	0.790	0.011	0.210	0.294	0.077	0.332	0.088	0.282	0.097	4.758
66	Kota	9	0.666	0.157	0.129	0.902	0.860	0.879	0.320	0.240	0.256	0.123	0.501	0.110	0.354	0.076	5.573
67	Kota	9	0.560	0.140	0.118	0.975	0.781	0.789	0.108	0.210	0.294	0.077	0.332	0.090	0.281	0.097	4.852
68	Kota	9	0.620	0.140	0.113	0.934	0.773	0.893	0.323	0.230	0.282	0.174	0.436	0.090	0.311	0.081	5.4
69[c,d]	Kota	9	0.400	0.089	0.074	0.561	0.500	0.500	0.186	0.130	0.016	0.079	0.289	0.050	0.254	0.060	3.188
70	Kota	9	0.523	0.119	0.095	0.845	0.652	0.660	0.010	0.190	0.226	0.118	0.344	0.070	0.244	0.077	4.173
71	Kota	9	0.430	0.093	0.080	0.580	0.573	0.590	0.228	0.164	0.161	0.107	0.400	0.065	0.361	0.063	3.895
72	Kota	9	0.760	0.228	0.200	1.360	0.247	0.183	0.342	0.279	0.430	0.072	1.410	0.100	0.940	0.168	6.719
73	Kota	9	0.415	0.090	0.077	0.564	0.556	0.573	0.221	0.160	0.156	0.144	0.384	0.060	0.350	0.060	3.81
74	Mandsaur	10	0.597	0.180	1.578	1.071	0.170	0.131	0.243	0.220	0.337	0.057	1.109	0.080	0.739	0.080	6.592
75[c]	Mandsaur	10	0.721	0.221	0.158	0.135	0.170	0.259	0.029	0.310	0.403	0.131	0.739	0.160	0.538	0.164	4.138
76	Mandsaur	10	0.768	0.246	0.212	1.265	0.200	0.268	0.031	0.330	0.434	0.197	0.859	0.150	0.841	0.154	5.955
77	Mandsaur	10	1.234	0.320	0.255	2.260	0.350	0.434	0.028	0.960	0.350	0.161	1.643	0.260	0.577	0.205	9.037
78	Mandsaur	10	0.682	0.202	0.183	1.109	0.140	0.220	0.025	0.270	0.350	0.132	0.764	0.120	1.000	0.138	5.335
79	Mandsaur	10	0.443	0.150	0.127	0.999	0.160	0.200	0.027	0.470	0.370	0.274	0.980	0.160	0.563	0.106	5.029
80	Mandsaur	10	0.689	0.211	0.189	1.120	0.220	0.197	0.292	0.280	0.409	0.177	0.750	0.120	1.068	0.132	5.854
81[c]	Mandsaur	10	0.778	0.194	0.245	1.487	0.170	0.252	0.285	0.520	0.458	0.035	0.828	0.170	0.098	0.025	5.545
82	Mandsaur	10	0.579	0.183	0.132	1.070	0.140	0.226	0.275	0.240	0.325	0.424	1.104	0.110	0.500	0.148	5.456
83	Mandsaur	10	0.526	0.174	0.152	1.097	0.160	0.133	0.241	0.220	0.287	0.343	0.990	0.110	0.615	0.015	5.063
84	Mandsaur	10	0.831	0.254	0.200	1.484	0.200	0.180	0.350	0.330	0.459	0.201	0.787	0.170	0.700	0.164	6.31
85	Mandsaur	10	0.725	0.238	0.212	1.353	0.220	0.220	0.340	0.300	0.440	0.223	0.813	0.130	0.881	0.132	6.227
86	Mandsaur	10	1.181	0.315	0.262	2.227	0.350	0.390	0.289	0.990	0.733	0.077	1.612	0.260	1.412	0.197	10.295
87[c]	Mandsaur	10	0.641	0.203	0.190	1.272	0.160	0.227	0.295	0.270	0.352	0.103	0.708	0.110	0.794	0.195	5.52
88	Mandsaur	10	0.597	0.187	0.176	0.985	0.210	0.154	0.279	0.250	0.360	0.080	0.692	0.120	0.854	0.014	4.958
89	Mandsaur	10	0.680	0.202	0.167	1.081	0.220	0.178	0.302	0.260	0.360	0.120	0.780	0.120	0.042	0.160	4.672
90	Mandsaur	10	0.828	0.250	0.198	1.700	0.220	0.240	0.305	0.560	0.613	0.078	1.218	0.160	1.276	0.145	7.791
91	Mandsaur	10	0.584	0.169	0.134	1.158	0.190	0.158	0.300	0.260	0.310	0.395	0.990	0.110	0.465	0.154	5.377
92[c]	Neemuch	11	0.649	0.192	0.164	1.180	0.160	0.220	0.035	0.390	0.320	0.096	0.908	0.150	0.815	0.097	5.376
93	Neemuch	11	0.937	0.241	0.262	1.750	0.260	0.250	0.028	0.520	0.664	0.086	1.360	0.170	1.338	0.171	8.037
94	Neemuch	11	0.547	0.162	0.125	0.961	0.160	0.145	0.259	0.210	0.231	0.022	0.593	0.110	0.237	0.147	3.909
95	Neemuch	11	0.931	0.104	0.237	1.633	0.250	0.230	0.262	0.090	0.183	0.057	1.245	0.170	1.197	0.133	6.732
96[c]	Neemuch	11	0.827	0.236	0.198	1.333	0.340	0.177	0.273	0.370	0.398	0.207	0.817	0.160	1.043	0.160	6.539
97	Neemuch	11	0.722	0.197	0.190	0.917	0.210	0.174	0.270	0.250	0.700	0.123	0.629	0.100	0.674	0.014	5.17
98	Neemuch	11	0.888	0.264	0.220	1.596	0.220	0.270	0.271	0.530	0.500	0.186	1.192	0.190	1.196	0.125	7.648
99[c,d]	Neemuch	11	0.772	0.210	0.203	1.297	0.200	0.230	0.02	0.510	0.311	0.377	1.303	0.170	0.938	0.184	6.725
100	Neemuch	11	0.952	0.930	0.218	1.720	0.260	0.230	0.022	0.510	0.540	0.089	1.098	0.190	0.702	0.147	7.581
101	Neemuch	11	0.543	0.173	0.130	0.895	0.170	0.140	0.260	0.220	0.294	0.019	0.885	0.110	0.761	0.115	4.715
102	Neemuch	11	0.840	0.229	0.195	1.506	0.280	0.180	0.330	0.330	0.433	0.201	0.851	0.160	1.045	0.150	6.73
103	Neemuch	11	0.268	0.075	0.076	0.474	0.070	0.148	0.104	0.100	0.134	0.067	0.228	0.040	0.304	0.042	2.13
104	Pratapgarh	12	0.698	0.188	0.194	1.104	0.180	0.190	0.102	0.100	0.135	0.015	1.001	0.140	0.801	0.069	4.917
105	Pratapgarh	12	0.355	0.082	0.103	0.504	0.100	0.100	0.105	0.200	0.262	0.013	0.604	0.080	0.550	0.054	3.112
106[c,d]	Pratapgarh	12	0.569	0.176	0.155	1.070	0.190	0.155	0.251	0.230	0.293	0.025	0.669	0.110	0.602	0.144	4.639
107	Pratapgarh	12	0.887	0.265	0.242	1.634	0.200	0.178	0.367	0.360	0.470	0.150	0.898	0.170	0.952	0.177	6.95
108	Pratapgarh	12	0.988	0.281	0.282	1.769	0.270	0.320	0.258	0.640	0.300	0.340	1.624	0.220	0.663	0.146	8.101
109	Pratapgarh	12	0.560	0.183	0.164	0.963	0.190	0.172	0.243	0.190	0.609	0.087	0.544	0.070	0.293	0.121	4.389
110	Tilhar	13	0.320	0.116	0.135	1.547	0.094	0.225	0.143	0.170	0.146	0.110	0.477	0.070	0.117	0.590	4.26
111	Tilhar	13	0.221	0.085	0.069	0.371	0.090	0.178	0.112	0.120	0.121	0.092	0.182	0.060	0.133	0.048	1.882
112	Tilhar	13	0.230	0.108	0.071	0.428	0.490	1.138	0.130	0.130	0.144	0.022	0.128	0.060	0.132	0.027	3.238
113[c,d]	Tilhar	13	0.374	0.161	0.134	0.870	0.140	0.634	0.231	0.210	0.312	0.144	0.502	0.090	0.365	0.093	4.26
114	Tilhar	13	0.215	0.090	0.062	0.393	0.450	1.010	0.118	0.100	0.187	0.037	0.139	0.060	0.215	0.045	3.121
115	Tilhar	13	0.247	0.106	0.056	0.339	0.107	0.072	0.053	0.160	0.107	0.074	0.257	0.050	0.137	0.919	2.684
116	Tilhar	13	0.334	0.088	0.091	0.546	0.091	0.186	0.129	0.100	0.155	0.058	0.286	0.060	0.208	0.064	2.396
117	Tilhar	13	0.196	0.070	0.060	0.453	0.065	0.230	0.101	0.090	0.118	0.055	0.246	0.040	0.166	0.041	1.931
118	Ujjain	14	0.630	0.194	0.135	1.151	0.150	0.150	0.241	0.260	0.368	0.150	0.580	0.150	0.365	0.060	4.584
119	Ujjain	14	0.992	0.286	0.235	1.640	0.280	0.340	0.252	0.870	0.682	0.150	1.344	0.210	1.370	0.163	8.814

Table 3 (*Continued*)

Sample	Division[b]	Group	D	T	S	E	G	A	V	I	L	Y	F	H	K	R	Total
120[c]	Ujjain	14	0.811	0.240	0.202	1.481	0.230	0.230	0.235	0.490	0.590	0.025	1.120	0.160	1.058	0.100	6.972
121	Ujjain	14	0.721	0.200	0.193	1.230	0.200	0.200	0.025	0.100	0.126	0.024	0.936	0.150	0.792	0.087	4.984
122	Ujjain	14	0.665	0.193	0.150	1.165	0.220	0.192	0.302	0.270	0.347	0.025	0.690	0.130	0.740	0.271	5.36
123	Ujjain	14	0.582	0.186	0.138	1.080	0.200	0.167	0.288	0.250	0.347	0.057	0.650	0.130	0.696	0.149	4.92
124	Ujjain	14	0.639	0.177	0.160	1.022	0.170	0.160	0.280	0.260	0.317	0.024	0.722	0.120	0.582	0.135	4.768

D: Aspartic acid; T: threonine; S: serine; E: glutamic acid; G: glycine; A: alanine; V: valine; I: isoleucine; L: leucine; Y: tyrosine; F: phenylalanine; H: histidine; K: lysine; and R: arginine.

[a] Values based on dry weight basis.

[b] For administrative purpose each state is divided into different divisions for licit opium cultivation. Thus, division column here indicates the area, where opium is legally cultivated.

[c] Opium samples analyzed for free amino acid profile.

[d] Opium samples analyzed for tryptophan, cystic acid and methionine sulfone.

Table 4
Percent composition (g%) of free pool of amino acids in Indian opium[a]

Serial no.	D	T	S	E	G	A	V	I	L	Y	F	H	K	R	Total FAA
1	ND[+]	0.08	0.143	0.015	ND	1.33	0.05	0.072	0.106	0.034	ND	0.14	0.157	ND	2.127
2	ND	0.045	0.14	0.018	ND	0.75	0.037	0.036	0.089	ND	ND	0.129	0.27	ND	1.514
3	0.035	0.03	0.03	ND	ND	0.347	0.024	0.038	0.107	ND	ND	0.063	ND	ND	0.7
4	ND	0.102	0.07	0.024	ND	2.12	0.067	0.106	0.09	0.034	ND	0.148	0.158	ND	2.933
5	0.1	0.023	0.081	ND	ND	0.22	0.033	0.04	0.039	0.022	ND	0.122	0.094	ND	0.774
6	0.063	ND	0.06	0.011	ND	0.385	0.033	0.059	0.041	ND	ND	0.126	0.169	ND	0.958
7	0.028	0.051	0.179	ND	ND	0.252	0.022	0.081	0.058	0.019	ND	0.134	0.428	ND	1.263
8	ND	0.027	0.023	ND	ND	0.452	0.021	0.03	0.019	ND	ND	0.093	0.034	ND	0.709
9	0.1	0.05	0.035	ND	ND	0.565	0.046	0.073	0.074	0.02	ND	0.105	0.03	ND	1.112
10	0.1	0.06	0.08	ND	ND	0.236	0.052	0.097	0.087	0.021	ND	0.132	0.166	ND	1.041
11	0.05	0.08	0.155	ND	ND	0.343	0.05	0.093	0.085	0.012	ND	0.152	0.43	ND	1.45
12	0.031	0.06	0.17	ND	ND	0.341	0.049	0.082	0.071	0.021	ND	0.14	0.421	ND	1.4
13	0.032	0.08	0.143	ND	ND	0.413	0.038	0.08	0.76	ND	ND	0.13	0.39	ND	2.066
14	0.024	0.053	0.168	ND	ND	0.186	0.031	0.07	0.05	ND	ND	0.12	0.3	ND	1.012
15	0.042	0.06	0.163	ND	ND	0.325	0.049	0.083	0.06	0.016	ND	0.138	0.41	ND	1.356
16	0.038	0.05	0.165	ND	ND	0.186	0.03	0.06	0.04	ND	ND	0.13	0.35	ND	1.049
17	0.05	0.07	0.14	ND	ND	0.43	0.037	0.065	0.062	0.065	ND	0.132	0.376	ND	1.49
18	0.024	0.038	0.043	0.021	ND	2.425	0.07	0.07	0.185	0.05	ND	0.148	0.167	ND	3.255
19	0.03	0.042	0.18	ND	ND	0.305	0.04	0.064	0.062	0.02	ND	0.132	0.36	ND	1.235

ND: Not detected if less than 0.01%; total FAA: total free amino acid composition (g%); D: aspartic acid, T: threonine, S: serine, E: glutamic acid, G: glycine, A: alanine, V: valine, I: isoleucine, L: leucine, Y: tyrosine, F: phenylalanine, H: histidine, K: lysine and R: arginine.

[a] Values based on dry weight basis.

a true discrimination between the licit opium growing areas of India. The mean and standard deviation of the amino acid variables were calculated between the groups as well as within the group. Variables that provide largest difference in means, but having smallest standard deviation within the group were selected. Variables that have same or very small difference among the means (<0.3) were ignored. The results obtained in the classification after this process of variable reduction were found to have a better predictive value of 90%. Further, same conclusion could be drawn even after the variable reduction process. The foremost amino acid variables that accounted for the true discrimination were identified as D, E, G, A, F and K (Fig. 4) in pin pointing the geographical origin of Indian opium. By the above study it is evident that only six amino acids are need to be determined for source identification of Indian opium. Based on the discriminant analysis one can clearly differentiate opium samples originating from the divisions of Madhya Pradesh and Pratapgarh (Rajasthan) from: (i) the divisions of Uttar Pradesh; and (ii) other divisions of Rajasthan (Fig. 4).

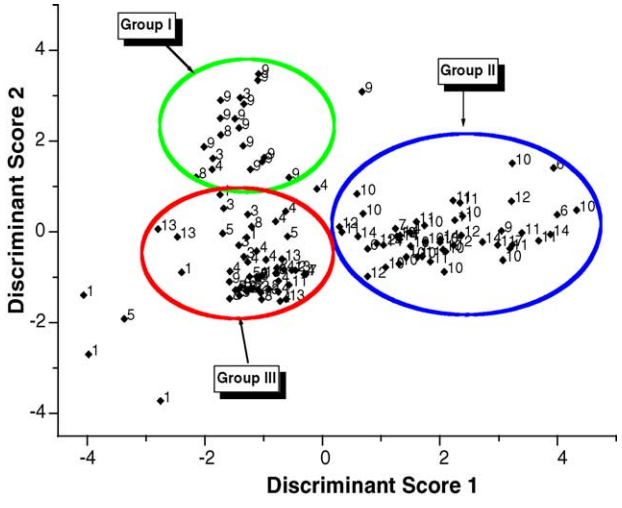

Fig. 4. Multivariate projection of the concentrations of six amino acids in the plane of two greatest discriminants for opium samples collected from licit opium growing areas of India. (1) Barabanki; (2) Bareilly; (3) Bhilwara; (4) Chittorgarh; (5) Faizabad; (6) Garoth; (7) Jaora; (8) Jhalwar; (9) Kota; (10) Mandsaur; (11) Neemuch; (12) Pratapgarh; (13) Tilhar; and (14) Ujjain.

Table 5
Percent composition (g%) of cystic acid in Indian opium samples

Sample	Amount of cystic acid found
1	0.214
2	0.168
3	0.164
4	0.145
5	0.132

A similar discriminant analysis was also performed on the data obtained by the analysis of free pool of amino acids in 19 opium samples. Based on the variable reduction process, five amino acid variables in free pool were found to contribute towards the discrimination of licit opium growing areas of India. Fig. 5 shows the multivariate projection of the five free pool of amino acids (D, T, S, A and V) in the plane of two greatest discriminant scores. The samples from Jaora (1/1), Mandsaur (3/3), Neemuch (3/3), Pratapgarh (1/1), Tilhar (1/1) and Ujjain (1/1) were found to segregate into one group (group I). The samples from Faizabad (1/1), Garoth (1/1) and Kota (2/2) were found to segregate into one group (group II). The samples from Barabanki, Bareilly, Bhilwara, Chittorgarh and Jhalwar were found to be outlyers in a two dimensional plot. In group I, the divisions except Pratapgarh and Tilhar belongs to the state of Madhya Pradesh. No clear separation of samples into groups was observed in case of discriminant analysis applied to free pool of amino acids as compared to the total amino acid profile (predictive

Table 6
Segregation of Indian opium samples into different groups based on the profile of six amino acids[a]

Serial no.	Division	State	No. of samples from each division	No. of samples falling in group I	No. of samples falling in group II	No. of samples falling in group III	No. of samples in outlyers
1	Barabanki	Uttar Pradesh	6			3	3
2	Bareilly	Uttar Pradesh	6			6	
3	Bhilwara	Rajasthan	8	2		6	
4	Chittorgarh	Rajasthan	15	1		13	1
5	Faizabad	Uttar Pradesh	6			5	1
6	Garoth	Madhya Pradesh	3		3		
7	Jaora	Madhya Pradesh	3		2	1	
8	Jhalwar	Rajasthan	9	2		7	
9	Kota	Rajasthan	17	14	1	1	1
10	Mandsaur	Madhya Pradesh	18		18		
11	Neemuch	Madhya Pradesh	12		11	1	
12	Pratapgarh	Rajasthan	6		6		
13	Tilhar	Uttar Pradesh	8			8	
14	Ujjain	Madhya Pradesh	7		7		
Total			124	19	48	51	6

[a] Amino acids include D, E, G, A, F and K.

Table 7
Reproducibility of retention times and peak areas of amino acids in opium samples after acid hydrolysis and after extraction with 80% aqueous ethanol

Serial no.	Amino acid	R.S.D. after acid hydrolysis (values in %)				R.S.D. after 80% aqueous ethanol extraction (values in %)			
		Retention time		Area		Retention time		Area	
		Intra[a]	Inter[a]	Intra	Inter	Intra	Inter	Intra	Inter
1	Cystic acid	0.05	0.23	2.34	3.31	0.07	0.34	3.14	3.43
2	Aspartic acid	0.14	0.67	2.56	3.05	0.18	0.45	2.67	3.51
3	Threonine	0.14	0.44	3.14	2.95	0.09	0.26	3.56	4.05
4	Serine	0.16	0.37	1.56	2.86	0.21	0.34	2.13	3.36
5	Glutamic acid	0.15	0.57	4.12	4.76	0.21	0.64	5.24	5.67
6	Glycine	0.13	0.42	4.31	3.96	0.17	0.47	4.45	6.43
7	Alanine	0.11	0.43	2.76	3.78	0.15	0.39	3.25	4.52
8	Valine	0.1	0.35	1.67	2.85	0.05	0.33	1.95	2.31
9	Isoleucine	0.09	0.32	3.39	4.13	0.06	0.27	4.02	4.54
10	Leucine	0.05	0.29	2.98	4.02	0.13	0.32	2.67	3.43
11	Tyrosine	0.95	1.12	4.96	4.86	0.88	0.89	4.34	5.04
12	Phenylalanine	0.85	1.14	3.56	3.87	0.37	0.53	3.12	4.32
13	Histidine	0.4	0.86	2.19	3.21	0.51	0.78	1.67	2.43
14	Lysine	0.19	0.56	3.15	3.97	0.32	0.85	3.75	4.57
15	Arginine	0.12	0.35	1.53	2.76	0.06	0.1	1.34	2.21

[a] $n = 5$, calculated from five independent measurements on the same day and between the days (7 days).

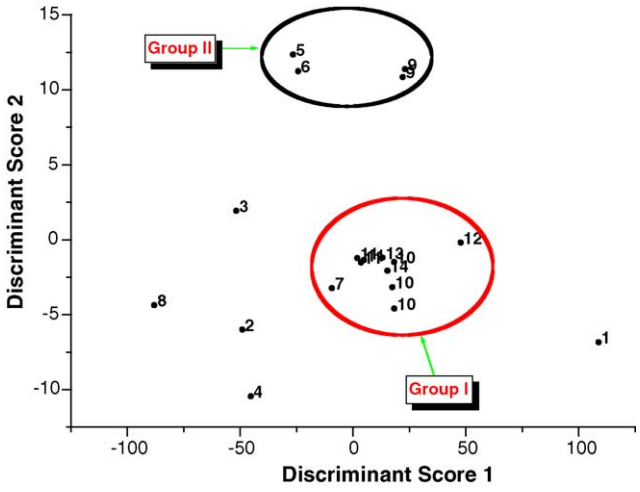

Fig. 5. Multivariate projection of the concentrations of five free pool of amino acids in the plane of two greatest discriminants for opium samples collected from licit opium growing areas of India. (1) Barabanki; (2) Bareilly; (3) Bhilwara; (4) Chittorgarh; (5) Faizabad; (6) Garoth; (7) Jaora; (8) Jhalwar; (9) Kota; (10) Mandsaur; (11) Neemuch; (12) Pratapgarh; (13) Tilhar; and (14) Ujjain.

value 90%). These observations suggest that use of data based on free pool of amino acid composition may not be useful in predicting the origin of the Indian opium samples.

Chromatographic fingerprinting of total amino acids in Indian opium samples along with the application of chemometrics affords an excellent tool in source identification of Indian opium. The methodology developed may further be used in discriminating the licit opium originating from countries such as Turkey and Australia.

4. Conclusions

Liquid chromatography with post-column *o*-pthalaldehyde derivatization methodology developed specifically for opium samples in the present investigations was found to be simple, highly sensitive, accurate and rapid as compared to the traditional chromatographic methods used earlier. The data based on the chromatographic fingerprinting of total amino acids gives an excellent analytical tool in predicting the origin of Indian opium samples. The present experimental investigation is a novel multidisciplinary approach, wherein chemometrics coupled with amino acid analytical data was successfully used as a tool in discriminating the licit opium growing areas of India. The methodology developed may find important forensic applications in: (i) discriminating Indian opium from other licit opium growing countries; and (ii) in pin pointing the source of seized opium originating from India.

Acknowledgements

The authors are thankful to Narcotic Commissioner, Ministry of Finance, GOI for providing the permission to collect the opium samples. The authors are also thankful to Chief Controller of Factories, New Delhi and General Managers, GOAW for providing the opium samples. The authors are grateful to Dr. J. Arunachalam, Head, CCCM, Hyderabad for the technical help provided in performing the chemometric analysis. The authors are also grateful to the Director, CFSL, Hyderabad for providing the necessary infrastructure facilities for carry out this work. Mr. M.M. Krishna Reddy is indebted to Directorate of Forensic Science, New Delhi for providing the research fellowship at CFSL, Hyderabad. Mr. M.M. Krishna Reddy is also indebted to CSIR, New Delhi for the award of Senior Research Fellowship to continue this study, at the Department of Biochemistry, Osmania University.

References

[1] Statistical Information on Narcotic Drugs, United Nations Office of Drug Control and Crime Preventive, 2003, p. 68.
[2] United States of America, National Drug Control Strategy, Reclaiming Our Communities from Drugs and Violence, Office of National Drug Control Policy, Executive Office of the President, Washington, DC, 1994, p. 16.
[3] Executive Summary on Global Illicit Drug Trends, United Nations Office on Drugs and Crime (UNODC), Vienna, 2003.
[4] The Assay, Characterization, Composition and Origin of Opium, United Nations Document Series ST/SOA/SER.K/1-148, 1951–1967.
[5] A. McKillop (Ed.), An Introduction to the Chemistry of the Alkaloids, Butterworths, London, UK, 1969, p. 139.
[6] L. Powden, Biol. Revs. 33 (1958) 393.
[7] J.C. Bartlet, C. Farmilo, Nature 174 (1954) 407.
[8] The Assay, Characterization, Composition and Origin of Opium, United Nations Documents, ST/SOA/SER.K/23, 30, 47, 63, 1951–1967.
[9] A. Jabber, E.B. Hanssen, UN Document ST/SOA/SER.K/102, 1960.
[10] S. Miyamoto, E. Brochmann-Hanssen, UN Documents ST/SOA/SER.K/109, 1960.
[11] B.N. Jones, J.P. Gilligan, J. Chromatogr. 266 (1983) 471.
[12] M. Roth, Anal. Chem. 43 (1971) 880.
[13] I.M. Perl, J. Chromatogr. A 913 (2001) 283.
[14] H.J. Liu, B.Y. Chang, H.W. Yan, F.H. Yu, X.X. Liu, J. AOAC Int. 78 (1995) 736.
[15] R. Schuster, J. Chromatogr. 431 (1988) 271.
[16] M. Fujiwara, Y. Ishida, N. Nimura, A. Toyama, T. Kinoshita, Anal. Biochem. 166 (1987) 72.
[17] Protein Quality Evaluation, Food and Agriculture Organization, Food and Nutrition Paper 51, Rome, Italy, 1991, p. 10.
[18] B.N. Jones, J.P. Gilligan, Am. Biotechnol. Lab. (1983) 46.
[19] L. Krenn, B. Kopp, B. Remberg, G. Buchbauer, A. Nikiforov, Pharmazie 49 (1994) 766.
[20] W.P. Gardiner (Ed.), Statistical Analysis, Methods for Chemists: A Software Based Approach, RSC press, Cambridge, UK, 1997, p. 313.