

Gas Chromatographic Determination of Amino Acids and Polyamines in Human Skin Samples using Trifluoroacetylacetone and Isobutyl Chloroformate as Derivatizing Reagents

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Abstract

An analytical method has been developed with improved sensitivity for the determination of amino acids and polyamines after precolumn derivatization with trifluoroacetylacetone and isobutyl chloroformate. 28 compounds comprising of 20 amino acids, five polyamines (1,3 diaminopropane, putrescine, cadverine, spermidine and spermine) and three biogenic compounds (4-aminobutyric acid, histamine and dopamine) separated completely with linear calibration range 1-10 µg/ml and limits of detection 60 -200 ng/ml. The separation was obtained within 11 min from HP-5 (30 m × 0.32 mm id) with column temperature 100°C for 2 min, followed by ramping at the rate of 20°C / min upto 250°C. The nitrogen flow rate was 3.0 ml/min. The method was applied for the analysis of the compounds in human skin samples after acid hydrolysis. The variation in their contents were examined in the affected skin samples from pemphigus vulgaris, psoriasis, leishmaniasis and eczema patients and result were compared with unaffected skin samples from healthy volunteers. The extraction of amino acids and polyamines from samples calculated by standard addition was within 95-102 % with RSDs 1.23-6.75 %.

Keywords: Gas chromatography; Amino acids; Polyamines; Pemphigus vulgaris; Psoriasis; Leishmania; Eczema; Healthy volunteers; Derivatization

Introduction

The human skin is self-renewing protective layer, providing the body with an impenetrable cover. It is composed of keratinocytes, which form an ordered array, including hair follicles and their appendages, such as sebaceous glands [1]. The keratinocytes are composed of protein molecules, which are synthesized from free amino acids precursors [2]. The information concerning their amino acids contents and their alterations as a result of pathological effects is limited [3]. The amino acids alteration from the normal value may be considered as a result of some disorder [4]. Thus the identification and determination of a number of amino acids may be useful for diagnosis of diseases including skin disorders [5].

The aliphatic amines of low molecular weight (putrescine, cadverine, spermidine and spermine) play a vital role in the synthesis of proteins and nucleic acids and also in the regulation of cell growth and proliferation [6]. Polyamines are also known to be the most important compounds for early diagnosis and treatment of cancer [7].

Pemphigus vulgaris (Pv) is a rare but sever blistering disease that affects the skin and mucous membrane [8]. Histopathologically Pv results in the formation of acantholysis of keratinocytes and blister in the suprabasal layer of epidermis. The binding of auto anti body to keratinocytes causes a subsequent loss of cell adhesion [9].

Atopic eczema is a chronic inflammatory skin disease that shows a wide variety of clinical pictures and is often complicated by relapse caused by different kinds of food [10]. The pathogenesis of eczema is reported due to a complex interaction of genetic predisposition and environmental factors [11].

Leishmaniasis is a disease caused by flagellated protozoan parasites of the genus leishmania, which belongs to the order kinetoplastida and family trypanosomatidae [12]. It is transmitted to their mammalian host by the bite of a hematophagous sandfly vector [13]. These parasites

are the causative agents of a broad spectrum of human diseases ranging from simple self healing cutaneous lesions to a severe visceral diminations [14].

Psoriasis is an inflammatory skin disease characterized by marked increases in keratinocytes proliferation, prominent alteration in dermal capillary vasculature and presence of dermal and epidermal T cells monocytes, macrophages and neutrophils [15,16].

The determination of amino acids and polyamines in biological materials continues to attract the attention of researches for physiopathological and clinical reasons. A large number of procedures have been developed for the determination of amino acids and polyamines but the procedures based on chromatographic methods and capillary electrophoresis [17-22] are more frequently reported. The analysis of amino acids by high performance liquid chromatography (HPLC) usually involve precolumn derivatization using various reagents including o-phthalaldehyde (OPA) [23], phenyl isothiocyanate [24], danyl chloride [25], or 9-fluorenylmethyl chloroformate (FMoC) [26]. The methods described include HPLC with photodiode array and fluorescence detection [27], HPLC-tandam mass spectrometry [28] and ultra high performance liquid chromatography tandem mass spectrometry [29]. However a number of difficulties have been reported to the procedures associated with HPLC and have been discussed [30]. The analysis of amino acids and polyamines by

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GC requires derivatization due to their polar nature. Different silyl derivatives or chloroformates are used as derivatizing reagents to bind amino and carboxylic acid groups simultaneously. Chloroformates as derivatizing reagents in GC have advantages over silylating reagents because of short reaction time in aqueous phase at room temperature [31-33]. The amino acids containing primary amino group react with the trifluoroacetylacetone (FAA) to form the derivatives amiable to GC column. The sensitivity has been enhanced by the derivatization of carboxylic group of amino acids with ethyl chloroformate [34]. The work also examines the GC of two stage derivatization using FAA and isobutyl chloroformate (IBCF) as reagents for further enhancement in sensitivity using FID detection. The method is applied for the determination of amino acids and polyamines contents in different human skin disorders after hydrolysis of proteins and compares the results with normal skin samples from healthy volunteers.

Experimental

Chemicals and solutions

The compounds glycine (Gly), L-alanine (Ala), L-valine (Val), L-phenylalanine (Phe), tryptophan (Trp), (Sigma, Louis, USA), tyrosine (Tyr), serine (Ser), proline (Pro), leucine (Leu), isoleucine (Ile), methionine (Met), threonine (Thr), (Sigma, Reichenhofen, Germany), glutamic acid (Glu), glutamine (Gln), aspartic acid (Asp), asparagine (Asn), cystine (Cys), lysine (Lys), histidine (His), arginine (Arg) (Sigma GmbH, Germany), 1,3- diaminopropane (1,3 Dap), putrescine (Put), cadaverine (Cad), spermidine (Spd), spermine (Spm), 4-aminobutyric acid (GABA), histamine (Hst) dopamine (Dop), trifluoroacetylacetone (FAA) and isobutyl chloroformate (IBCF) (Fluka Buchs, Switzerland), methanol (Rdh, Chemicals Co.Springs valley, CA, USA) were used.

Guaranteed reagent grade hydrochloric acid (37%), potassium chloride, acetic acid, sodium acetate, ammonium acetate, sodium tetraborate, boric acid, sodium bicarbonate, sodium carbonate, ammonium chloride and ammonia solution were from E.Merk, Darmstadt, Germany.

Stock solutions of amino acids, polyamines and other biological active compounds containing 1 mg/ml were prepared in methanol and water. Further solutions were prepared by appropriate dilution. Buffer solutions (0.1 M) between pH 3-10 at unit and 0.5 unit were prepared from the following: acetic acid-sodium acetate (pH 3-6), ammonium acetate (pH 7), boric acid-sodium tetraborate (pH 7.5-8.5), sodium bicarbonate-sodium carbonate (pH 9), ammonium chloride-ammonia solution (pH 10).

Equipments

The pH measurements were made with an Orion 420A pH meter (Orion Research Inc. Boston, USA) with combined glass electrode and reference internal electrode, GC studies were carried out on an Agilent model 6890 network GC system, connected with flame ionization detector (FID) and split injector (Agilent Technologies Santa, Clara, CA, USA), hydrogen generator (Parker Balson) Analytical Gas system (H-90, Parker Hannif Haverhill, MA, USA) and pure nitrogen (British oxygen company (BOC) Karachi, Pakistan), computer with Chemstation software controlled the gas chromatograph. Capillary column HP-5 (30 m x 0.32 mm id) with film thickness 0.25 μ m (J. W Scientific GC column Wilmington, Nc, USA) was used throughout the study.

GC Analytical procedure

The solution (0.2-3.0 ml) containing a mixture of amino acids,

polyamines, GABA, Hst and Dop (1-10 μ g each) was added to 0.3 ml ammonium acetate buffer pH 7 and 0.3 ml FAA (2% v/v in methanol). The contents were heated on water bath (95°C) for 20 min. The mixture was allowed to cool at room temperature and 0.3 ml of solvent system (acetonitrile-water-methanol-pyridine 42:42:8:8 v/v) was added to it. The mixture was then added to 0.3 ml IBCF (2% in methanol) and carbonate buffer pH 9 (0.3 ml). The mixture was sonicated at room temperature (30°C) for 15 min. Chloroform (1.0 ml) was added and contents were mixed well. The layers were allowed to separate and an aliquot of organic layer was transferred to screw capped sample vial. The solution (1 μ l) was injected in GC with split ratio 10:1 on the column HP-5 (30 m x 0.32 mm id) with film thickness 0.25 μ m at column temperature 100°C for 2 min., followed by ramping at the rate of 20°C/min up to 250°C and maximum temperature was maintained for 2 min. The nitrogen flow rate was 3 ml/min. The injector and detector temperatures were 270°C and 280°C respectively. The flow rates for FID were fixed for nitrogen as make up gas 45 ml/min, hydrogen 40 ml/min and air 450 ml/min.

Skin samples analysis

Dried skin sample (0.5 g) was added to 6 N hydrochloric acid (5 ml) in screw capped sample vial and contents were heated at 110°C for 24 h. The mixture was cooled and centrifuged for 20 min at 3000 g. The clear supernatant layer was separated and the residue was washed with de-ionized water (1 ml). The solvent from the combined extract was evaporated gently under nitrogen atmosphere. The residue was dissolved in water and volume was adjusted to 10 ml. The solution (3.0 ml) was taken and GC analytical procedure was followed. The quantitation was made from external calibration curve prepared from linear regression equation $Y=ax + b$.

Analysis of amino acids from skin samples using linear calibration curve with spiked samples

The dried skin sample (0.5g) from healthy volunteer, pemphigus vulgaris, leishmaniasis, psoriatic and eczema patients was treated as "skin samples analysis". Two portions of 3 ml from each of the sample were taken and a portion was processed as GC analytical procedure and the other was added to the solution of amino acids and polyamines (0.5 ml) containing 10 μ g/ml each and GC analytical procedure was again followed. The quantitations were carried out from external calibration curves and from increases in the responses with added standards.

Procedure for sample collection

The skin's cut pieces during circumcision of normal children ages between 5 and 10 years with verbal/written permission of the parents of the children, were taken from Liaquat University of Medical and Health Sciences (LUMHS) Hospital, Hyderabad and were placed in separate screw capped tube containing formaldehyde solution (20%). New hand gloves were used for the collection of each sample. The skin pieces were washed thoroughly with de-ionized distilled water in the laboratory and dried at 60°C for 45 min. Alternatively, the feet of healthy volunteers who had not taken any medicine at least one preceding week with verbal/written consent were washed with de-ionized water and methanol and allowed to dry. The upper layer of the feet was collected by rubbing it gently with iron gauze pre washed with de-ionized water and methanol. Each of the samples was transported to the laboratory separately and was oven dried at 60°C for 45 min.

The skin samples of patients where possible were obtained by applied biopsy. The skin was washed thoroughly with de-ionized water and methanol. The punches were applied 4-8 mm deep at the lesions

of the patients and were rotated clockwise and anti clockwise to cut the pieces of the skin. These pieces of the skin were placed separately in screw capped test tubes containing formaldehyde solution (20%) and were transferred to the laboratory for analysis. Each of the samples was thoroughly washed with de-ionized water in the laboratory and was dried at 60°C for 45 min.

In case of Pv, P and E the scales on the surface of the skin were also scratched with new surgical blade for each patient. To obtain the skin sample of L the xylocaine solution was injected at the infected part of the patient and then applied punch biopsy. The collected samples were placed in formaldehyde 20% solution and then transported to the laboratory for analysis.

The patients suffering from pemphigus vulgaris, psoriasis, leishmaniasis and eczema were identified by the consultants of Dermatology wards of LUMHS, Hyderabad Hospital and Shaheed Mohatarma Benazeer Bhutto Medical University Hospital Larkana, Sindh, Pakistan.

Results and Discussion

The reagent FAA is known to react with primary amino groups of amino acids, polyamines and biological active compounds to form highly stable Schiff bases [34,35]. The reagent IBCF is also reported to react with carboxylic group of amino acid and to secondary amino groups to form derivative with extension of carbon chain length [36] and increase in the sensitivity of FID detection. Both the reactions are carried out in aqueous medium. The optimum derivatization conditions of amino acids with FAA and polyamines and GABA, Hst and Dop were checked as reported [34], followed by with IBCF. The reactions were monitored by GC and the condition which gave maximum response (peak height/peak area) was considered optimum. The reaction conditions were examined in terms of the effect of pH, concentration of derivatizing reagent, reaction time and temperature and reaction medium. The maximum reaction of FAA was observed at pH 7 with ammonium acetate buffer with 0.3 ml of (2% FAA in methanol) per determination as derivatizing reagent and warming time of 20 min at 95°C.

The amino acids after derivatization with FAA were examined for second derivatization with IBCF and an enhancement in GC-

FID response for each of the compound was observed and thus reaction conditions were optimized. The reactions of chloroformates are reported in alkaline medium and therefore the effect of pH was examined within 6-12 at 0.5 and unit interval. The maximum response was observed with carbonate buffer pH 9 and was selected. The volume of carbonate buffer was varied from 0.2 ml to 1.0 ml at an interval of 0.1 ml. The addition of the different volumes of the buffer was not significant and addition of 0.3 ml was selected. The addition of IBCF (2% v/v in methanol) was varied from 0.1 to 0.6 ml at an interval of 0.1 ml and it was observed that the addition of IBCF was not critical as long excess of IBCF was available in the reaction medium. The addition of 0.3 ml was selected. The addition of the solvents for reaction medium was examined. Water, water-methanol, water-acetonitrile, water-pyridine base and solvent system proposed by Husek [37] (acetonitrile-water-methanol-pyridine 42:42:8:8 v/v) were investigated. The solvent system proposed by Husek proved better and was used. The volume of the solvent was varied between 0.2-1.0 ml at an interval of 0.1 ml and the effect of different volumes of solvent was not significant and 0.3 ml was used. The solvent chloroform, amyl alcohol and ethyl acetate were examined for the extraction of the derivatives and chloroform proved better and was used [37]. The amino acids, polyamines and biogenic amines derivatives after their formation and after different intervals of time were examined on GC and no change in the response was observed upto 24 h.

The amino acids, polyamines, GABA, Hst and Dop derivatives formed, after necessary extraction in chloroform was examined for GC separation using different temperature elution programmes. Finally complete and base line separation of all 28 compounds examined was found from HP-5 (30 m x 0.32 mm id), when eluted at column temperature 100°C for 2 min, followed by heating rate 20°C/min upto 250°C. The maximum temperature was maintained for 2 min. The total run time was 11.5 min. The nitrogen flow rate was maintained at 3 ml/min. The order of the elution of amino acids was Gly, Ala, 1,3 Dap, Put, Ser, Cad, Pro, GABA, Val, Thr, Leu, Ile, Cys, Asn, Asp, Hst, Lys, Gln, Glu, Met, Spd, His, Phe, Dop, Arg, Tyr, Trp, Spm (Figure 1). The capacity factor (K') calculated for the amino acids varied within the range 0.87-5.87. The separation was repeatable in terms of retention time and peak height/peak area with RSD's (n=5) within 0.96-3.33 % and 2.30-4.84 % respectively.

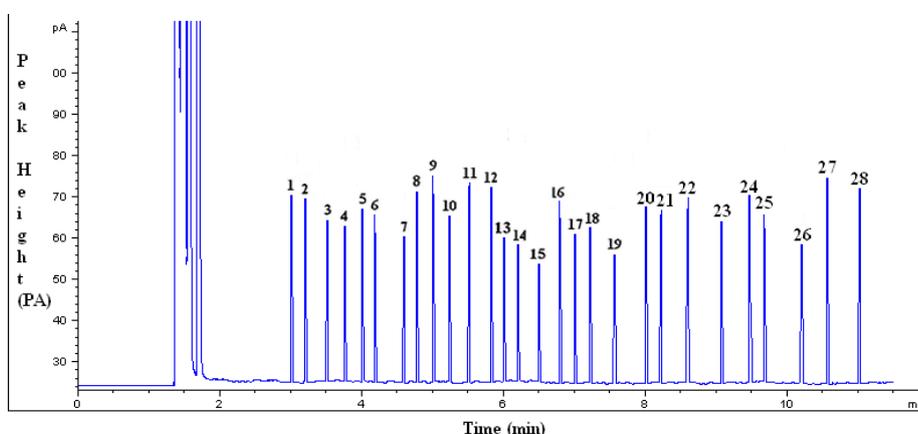


Figure 1: Separation of amino acids and polyamines using FAA and IBCF as derivatizing reagents. 1 Gly, 2 Ala, 3 1,3 Dap, 4 Put, 5 Ser, 6 Cad, 7 Pro, 8 GABA, 9 Val, 10 Thr, 11 Leu, 12 Ile, 13 Cys, 14 Asn, 15 Asp, 16 Hst, 17 Lys, 18 Gln, 19 Glu, 20 Met, 21 Spd, 22 His, 23 Phe, 24 Dop, 25 Arg, 26 Tyr, 27 Trp, 28 Spm. GC conditions: column HP-5 (30 m x 0.32 mm id) with film thickness of 0.25 μ m at column temperature 100 °C for 2 min with ramping of 20 °C/min up to 250 °C and stay at maximum temperature for 2 min. Nitrogen flow rate of 3 ml/min with split ratio 10:1. The injector and detector temperatures were 270 °C and 280 °C, respectively.

Amino Acid	LOQ (µg mL ⁻¹)	LOD (µg mL ⁻¹)	Retention time (min)	Linear calibration range	Coefficient of determination (r ²)	Linear regression equation
Gly	0.18	0.06	3	1-10	0.9983	y=4.53x + 0.02
Ala	0.18	0.06	3.2	1-10	0.9999	y=4.405x + 0.17
1,3 Dap	0.18	0.06	3.5	1-10	0.9962	y=3.8357x + 0.0558
Put	0.18	0.06	3.8	1-10	0.9995	y=3.6979x + 0.2659
Ser	0.31	0.1	4	1-10	0.9992	y=4.145x - 0.05
Cad	0.18	0.06	4.2	1-10	0.9984	y=3.9778x + 0.0793
Pro	0.31	0.1	4.6	1-10	0.9933	y=3.41x + 0.24
GABA	0.18	0.06	4.8	1-10	0.9991	y=4.5704x + 0.2958
Val	0.31	0.1	5	1-10	0.9912	y=4.975x + 0.45
Thr	0.31	0.1	5.3	1-10	0.9946	y=3.92x + 0.34
Leu	0.61	0.2	5.5	1-10	0.9959	y=4.765x + 0.11
Ile	0.61	0.2	5.8	1-10	0.9993	y=4.64x + 0.02
Cys	0.18	0.06	6	1-10	0.9978	y=3.525x - 0.03
Asn	0.18	0.06	6.2	1-10	0.9998	y=3.235x + 0.17
Asp	0.18	0.06	6.5	1-10	0.9965	y=2.835x + 1.21
Hst	0.31	0.1	6.8	1-10	0.9945	y=4.3707x - 0.6397
Lys	0.31	0.1	7	1-10	0.9919	y=3.65x - 0.2
Gln	0.31	0.1	7.3	1-10	0.9992	y=3.72x + 0.02
Glu	0.18	0.06	7.6	1-10	0.9999	y=3.03x + 0.1
Met	0.61	0.2	8	1-10	0.9968	y=4.23x + 0.04
Spd	0.31	0.1	8.2	1-10	0.9953	y=4.1267x + 0.49
His	0.61	0.2	8.6	1-10	0.9966	y=4.305x + 0.07
Phe	0.31	0.1	9.1	1-10	0.9987	y=3.825x - 0.01
Dop	0.61	0.2	9.4	1-10	0.9933	y=4.3824x + 0.069
Arg	0.31	0.1	9.7	1-10	0.998	y=4.01x + 0.54
Tyr	0.31	0.1	10.2	1-10	0.9995	y=3.325x + 0.07
Trp	0.61	0.2	10.6	1-10	0.9977	y=4.85x + 0.62
Spm	0.61	0.2	11	1-10	0.9949	y=4.5971x + 0.9761

Table 1: Analytical data for the amino acids and polyamines analysis using FAA and IBCF as derivatizing reagents.

Reagent	Compounds analyzed	Limit of detection (LOD)	Limit of quantitation (LOQ)	Caliberation range	Retention time (min)	Detection	Reference
BSTFA	16	0.01-4.24 µmol/l	0.02-7.07 µmol/l	0.1-133 µmol/l	17	GC-MS	21
Propyl chloroformate	18	0.5-1.0 µmol/l	0.9-3.0 µmol/l	0.3-1072 µmol/l	20	GC-MS	28
FAA+ECF	19	0.1-0.3 µg/ml	0.31-0.91 µg/ml	1-10 µg/ml	10	GC-FID	34
FAA+IBCF	28	0.06-0.2 µg/ml	0.18-0.61 µg/ml	1-10 µg/ml	11	GC-FID	Present

Table 2: Comparative GC analysis of amino acids and polyamines by reported procedures.

The linearity of the calibration curves were checked for all the 28 compounds separated by recording average peak height/peak area (n=4) verses concentration and linear relationship was observed within 1-10 µg/ml for each of the compound with coefficient of determination within 0.9912-0.9999 using 6 calibrators. The limits of detection (LOD) measured as signal to noise ratio 3:1 were calculated within 60-200 ng/ml, corresponding to 6-20 pg injected on the column with split ratio 10:1. The limit of quantitation (LOQ) measured as signal to noise ratio (10:1) was calculated 0.18-0.61 µg/ml. The linear regression equations for the amino acids were calculated and are summarized in Table 1. The repeatability of the derivatization, separation and quantitation was examined inter (n=5) and intra (n=5) day by the same operator at the concentration 5 µg/ml in terms of retention time and peak height/peak area and RSD were within 0.96-3.33 % and 2.29-5.25 % respectively. The accuracy of the analysis of test mixtures (n=4) of amino acids within the calibration range was checked and the relative error was obtained within ± 02-03 %.

de Paiva et al have reported GC separation of 16 amino acids as N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) derivatives within 17 min [21]. Kasper et al. separated 18 L-amino acids by GC as propyl

chloroformate derivative with run time of 20 min [28]. The present work reports separation of 28 compounds with 11 min (Table 2).

Now comparing the GC using FAA and ECF as derivatizing reagent [34], it was observed an increase in the retention time with an improvement in the FID sensitivity for the analysis of amino acids using FAA and IBCF as derivatizing reagent due to an increase in carbon numbers. A change in elution order was also observed when using FAA and IBCF as compared to FAA and ECF as derivatizing reagent, may be due to the effect of bulky isobutyl instead of ethyl group (Table 2).

Sample analysis

The developed method was applied for the analysis of the compounds from acid hydrolyzed human skin samples. Skin samples collected from 10 healthy volunteers (H) and 8 each of Pv, P, L, and E patients were analyzed. The peak identification was made based on comparing the retention time of the standards. 23 compounds comprising 20 amino acids and three polyamines (Put, Spd, Spm) were identified from all the samples analyzed (Figure 2). The results of analysis are summarized in Table 3. The average values with standard deviation and range (minimum- maximum values are summarized in

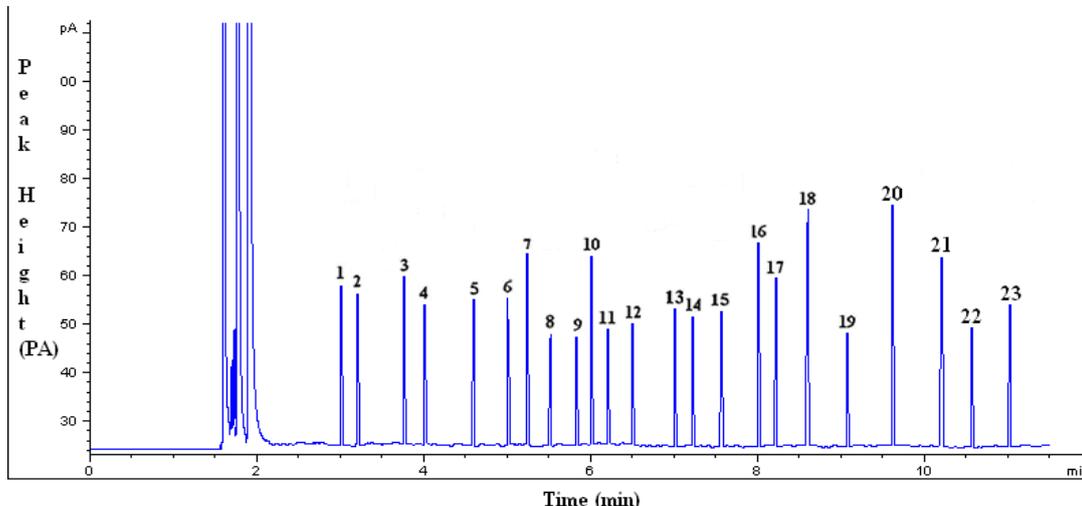


Figure 2: Quantitative response of amino acids and polyamines from leishmania patient using FAA and IBCF as derivatizing reagents. 1 Gly, 2 Ala, 3 Put, 4 Ser, 5 Pro, 6 Val, 7 Thr, 8 Leu, 9 Ile, 10 Cys, 11 Asn, 12 Asp, 13 Lys, 14 Gln, 15 Glu, 16 Met, 17 Spd, 18 His, 19 Phe, 20 Arg, 21 Tyr, 22 Trp, 23 Spm. GC conditions as fig. 1

	Gly	Ala	Put	Ser	Pro	Val	Thr	Leu	Ile	Cys	Asn	Asp	Lys	Gln	Glu	Met	Spd	His	Phe	Arg	Tyr	Trp	Spm
H1	65 (1.6)	65.1 (1.5)	45 (1.6)	92.4 (0.8)	77.9 (1.4)	21.5 (0.7)	37.3 (1.4)	54.7 (0.9)	55.6 (0.8)	42.4 (1.7)	34 (1.4)	27 (1.5)	45.3 (1.3)	33.8 (1.3)	29.5 (0.8)	36.8 (1.0)	39.6 (0.7)	58.1 (1.3)	30.3 (1.6)	48.3 (1.3)	36.7 (1.6)	19.3 (1.4)	35.1 (1.5)
H2	69.6 (0.9)	69.8 (1.4)	48.7 (1.3)	95.3 (1.4)	77 (1.5)	20.4 (1.2)	36.6 (1.6)	52.7 (0.9)	52.4 (1.4)	46.8 (1.3)	29.5 (0.8)	21.8 (1.5)	43.3 (0.8)	29.5 (0.7)	25.7 (1.4)	35.7 (1.5)	43.8 (0.9)	59.4 (1.3)	31.7 (1.0)	46.3 (1.8)	40.7 (1.8)	23.1 (1.6)	37.7 (2.0)
H3	66.3 (0.9)	66.5 (0.8)	53.4 (0.9)	94.5 (1.2)	76.6 (0.8)	23.2 (0.9)	31.9 (1.5)	53.4 (0.8)	50.5 (1.1)	40.7 (1.8)	35.3 (1.3)	19 (1.3)	42.2 (1.0)	30.8 (0.9)	26.8 (1.3)	40.6 (1.0)	46.5 (0.8)	55.5 (1.2)	27.7 (0.8)	44.3 (1.2)	39.1 (1.6)	26.1 (1.7)	41.5 (0.8)
H4	68.7 (1.0)	68.9 (1.6)	46.9 (1.2)	97.7 (1.3)	81.1 (1.5)	18.8 (1.4)	34.4 (1.1)	56.5 (0.9)	56.9 (1.5)	44.5 (0.8)	32.4 (0.9)	25.1 (0.9)	42.6 (0.9)	26.7 (1.2)	23.1 (1.4)	41.2 (1.4)	43.3 (0.8)	57 (1.7)	28.4 (0.7)	53 (1.5)	44.6 (1.1)	22.2 (1.5)	37.7 (1.2)
H5	70.2 (1.0)	70.4 (0.9)	45.3 (1.3)	93 (1.4)	83 (1.3)	21.2 (1.4)	36.5 (1.0)	51.5 (0.9)	54.3 (1.4)	41.5 (0.8)	34 (1.6)	27 (1.6)	44.9 (1.5)	28.1 (1.3)	24.9 (1.5)	40.3 (1.3)	40.4 (1.2)	58.9 (1.3)	24.1 (1.4)	48.3 (1.3)	41.1 (1.5)	20.3 (1.6)	35.3 (1.3)
H6	66.9 (1.4)	67.1 (1.1)	53.2 (1.3)	96.7 (1.6)	75.9 (1.4)	23.1 (1.4)	32.6 (1.1)	54.7 (0.9)	54.7 (0.9)	44.5 (0.7)	35.3 (1.3)	19 (1.4)	46.8 (1.3)	33.3 (0.7)	29.9 (1.5)	37.9 (1.2)	46.4 (0.8)	58.4 (1.0)	30.5 (0.9)	50.3 (0.9)	43.3 (0.8)	26.1 (1.2)	40.8 (1.3)
H7	65.9 (1.7)	66 (1.4)	47.5 (0.7)	95.6 (1.5)	78.1 (1.3)	18.9 (1.6)	37.3 (2.0)	52.3 (0.9)	53 (1.5)	43.4 (0.9)	29.1 (1.7)	26.3 (1.3)	40.2 (1.4)	23.4 (0.8)	27.5 (1.1)	41.7 (1.4)	42.2 (1.7)	61.2 (1.3)	26.9 (1.4)	47.1 (1.5)	38.5 (1.1)	22.2 (1.5)	37 (1.6)
H8	68.1 (1.1)	68.3 (0.9)	48.2 (1.1)	93.8 (1.5)	76.5 (0.7)	20.6 (0.9)	34.9 (1.4)	56.5 (0.9)	51.3 (1.0)	41.1 (1.5)	31.8 (0.9)	24.4 (0.8)	43.3 (0.8)	27.4 (0.9)	22.8 (0.9)	35.2 (1.2)	43.1 (1.5)	54.4 (0.7)	31.7 (1.0)	49.3 (0.8)	44.4 (1.3)	22.9 (1.4)	38.3 (2.1)
H9	69.7 (1.2)	70 (1.7)	46.9 (0.4)	91.3 (0.9)	78.1 (1.3)	22.8 (0.9)	36.5 (1.1)	51.2 (1.6)	55.1 (1.5)	43.7 (1.4)	35.1 (1.5)	21.1 (1.5)	45.5 (0.9)	31.3 (0.9)	28.8 (1.2)	36.5 (1.0)	41.5 (0.9)	56.4 (1.1)	29.5 (0.7)	48 (1.3)	41.7 (1.4)	21.4 (1.4)	37 (1.4)
H10	67.2 (1.3)	66.6 (0.7)	42.9 (1.9)	92.7 (1.0)	77.3 (1.6)	22.4 (1.1)	32.2 (1.1)	52.4 (0.7)	53.4 (1.4)	39.2 (1.0)	28.1 (1.6)	20.2 (1.3)	37.4 (0.8)	24.5 (0.7)	22 (1.4)	37.6 (1.0)	37.5 (0.9)	52.9 (1.6)	23 (1.4)	43.3 (0.8)	35.1 (1.5)	18.7 (1.5)	33.4 (0.7)
H10a	66.8 (1.4)	66.2 (1.5)	41.8 (1.5)	93.2 (1.4)	76.8 (1.2)	21.5 (0.7)	31.8 (0.9)	52.5 (0.9)	53.1 (1.3)	38.3 (2.1)	26.8 (0.4)	20.5 (1.3)	36.3 (1.2)	23.3 (0.8)	21.7 (1.3)	36.6 (1.3)	36.5 (1.0)	51.8 (1.2)	22.9 (1.7)	42 (1.7)	34.2 (1.6)	17.8 (1.5)	32.6 (1.1)
MEAN	67.8	67.9	47.8	94.3	78.2	21.3	35.0	53.6	53.7	42.8	32.5	23.1	43.2	28.9	26.1	38.4	42.4	57.2	28.4	47.8	40.5	22.2	37.4
SD	1.78	1.86	3.45	2.03	2.22	1.62	2.14	1.93	1.98	2.24	2.74	3.23	2.78	3.50	2.87	2.40	2.85	2.49	3.01	2.81	3.18	2.51	2.48
PV1	59.3 (0.9)	60.4 (1.2)	64.4 (1.2)	78.4 (0.9)	69 (1.4)	34 (1.4)	52.8 (1.2)	44 (1.4)	46.5 (0.9)	55.7 (1.5)	66.4 (1.3)	43.5 (0.8)	62.8 (1.8)	21.1 (1.5)	47.7 (1.4)	52.1 (1.4)	57.2 (1.4)	66.2 (0.9)	20.6 (0.9)	61.6 (1.4)	51.4 (1.1)	34.5 (0.8)	50.8 (0.9)
PV2	61.3 (1.1)	59.8 (1.3)	64.1 (1.4)	79.2 (1.6)	66.1 (1.6)	34.5 (0.8)	49.7 (0.9)	42.4 (1.7)	48.5 (1.7)	58.3 (0.9)	69.3 (0.7)	45.1 (1.3)	60.5 (0.8)	18.8 (1.4)	45.1 (1.3)	54.3 (1.4)	57.5 (1.1)	68.2 (1.6)	23.5 (1.8)	62.9 (1.7)	52.6 (0.9)	35.3 (1.3)	50.5 (1.1)
PV3	59.1 (1.5)	57.7 (1.4)	64.4 (0.7)	77.1 (1.2)	73.9 (1.8)	33.7 (1.3)	51.8 (1.2)	41.5 (0.8)	45.8 (2.2)	56.6 (0.8)	65.4 (0.6)	40.6 (0.8)	61.9 (1.3)	22.7 (1.8)	48.4 (0.7)	51.6 (0.9)	56.6 (0.8)	67.1 (1.1)	21.5 (0.7)	59.8 (1.3)	51.6 (0.6)	34.6 (0.8)	50.8 (0.9)
PV4	58.7 (1.7)	61 (1.2)	61.7 (0.7)	79.4 (0.8)	71.2 (1.8)	31.7 (1.0)	52.6 (0.9)	42.5 (1.9)	46.8 (1.3)	56.6 (1.4)	66.8 (1.4)	44.9 (1.5)	59.7 (1.4)	18.4 (0.8)	46.6 (1.1)	53 (1.5)	55.3 (1.3)	65.9 (1.7)	20.4 (1.2)	62.6 (0.6)	49.4 (0.8)	33.1 (1.7)	48.6 (1.0)
PV5	60.7 (1.2)	59.1 (1.5)	65.5 (0.9)	77.9 (1.4)	67.1 (1.1)	34.9 (1.4)	51.3 (1.1)	43.5 (1.9)	45.2 (1.3)	59.3 (0.9)	64.5 (0.8)	42.3 (1.3)	61 (1.2)	20.4 (1.2)	49.3 (0.8)	51.2 (1.6)	56.7 (1.6)	67.3 (1.5)	23 (1.3)	61.3 (1.1)	52.2 (1.5)	35 (1.3)	51.7 (1.5)
PV6	61.9 (1.3)	62.4 (0.9)	59.9 (1.6)	80.8 (1.1)	72.6 (1.1)	36.1 (1.4)	49.9 (1.5)	45.6 (0.9)	49.1 (1.5)	61.5 (1.3)	68.5 (0.9)	47.7 (1.4)	65.4 (0.6)	22.4 (1.2)	45.5 (0.9)	54.5 (1.2)	53.7 (0.9)	69.9 (1.4)	22.2 (1.5)	59.3 (0.9)	50.6 (0.9)	31.8 (0.9)	47.2 (1.4)

PV7	59.1 (1.5)	58.5 (1.3)	63 (1.9)	76.6 (0.8)	68.6 (0.8)	33.7 (1.3)	55.2 (1.2)	42.5 (0.9)	46.5 (1.1)	58.3 (0.9)	71.1 (1.4)	39.9 (1.5)	60.3 (0.9)	24.7 (1.2)	51.7 (1.0)	56.4 (1.1)	57 (1.4)	69.3 (0.7)	25.5 (0.7)	58.8 (1.6)	46.8 (1.8)	33.9 (1.8)	49.6 (1.7)
PV8	58.5 (1.2)	60.1 (1.9)	65.7 (0.6)	79.4 (0.8)	72.6 (0.8)	32.6 (1.1)	53 (1.4)	43.6 (0.9)	47.8 (1.8)	55.7 (1.1)	67.4 (1.5)	45.8 (2.2)	63.4 (0.9)	20.8 (1.2)	46.9 (1.2)	52.7 (1.2)	59 (1.5)	66.6 (1.5)	19.5 (1.2)	61.4 (1.5)	49.6 (0.6)	35.8 (1.6)	51.8 (1.2)
PV8a	58.7 (1.7)	60 (1.3)	64.7 (1.6)	79.3 (1.4)	72.5 (0.7)	31.7 (1.0)	52.4 (1.4)	44.1 (1.6)	47.5 (0.7)	54.6 (1.1)	66.8 (1.4)	45.7 (1.4)	62.3 (1.0)	20 (1.5)	46.4 (0.8)	51.4 (1.1)	58.7 (1.6)	66.2 (0.9)	18.9 (1.6)	60.5 (1.1)	48.8 (1.4)	34.6 (0.8)	51 (1.3)
MEAN	59.8	59.9	63.6	78.6	70.1	33.9	52.0	43.2	47.0	57.8	67.4	43.7	61.9	21.2	47.7	53.2	56.6	67.6	22.0	61.0	50.5	34.3	50.1
SD	1.29	1.47	1.97	1.38	2.84	1.35	1.79	1.26	1.34	2.00	2.15	2.67	1.91	2.08	2.15	1.74	1.59	1.45	1.95	1.51	1.89	1.29	1.58
P1	54.1 (1.1)	54.5 (1.2)	68.4 (1.9)	66.8 (1.4)	63.1 (1.2)	26.7 (1.2)	74.6 (0.9)	36.8 (1.2)	40.8 (1.3)	35.2 (0.9)	56.3 (0.6)	64.2 (1.4)	37 (1.6)	60.4 (1.2)	69.5 (0.7)	30.8 (0.9)	60.8 (1.8)	45.9 (1.6)	49.5 (0.7)	42.8 (1.5)	32.5 (0.8)	38 (2.2)	54 (1.5)
P2	51 (1.3)	55.9 (1.6)	66.6 (1.5)	63.9 (1.4)	61.9 (1.4)	28.7 (1.1)	77.1 (1.3)	35.8 (1.6)	41.8 (1.5)	30.7 (0.9)	54.9 (1.1)	62.5 (0.8)	33.6 (1.1)	58 (1.3)	66.7 (1.5)	32.6 (1.1)	58.8 (1.6)	42.6 (0.9)	51.8 (1.4)	40.2 (0.9)	29.7 (0.7)	36.1 (1.4)	52.5 (1.0)
P3	52.3 (0.9)	54.1 (1.1)	67.5 (1.2)	64.7 (1.2)	61.4 (1.5)	25.2 (1.4)	73.9 (1.8)	36.3 (1.2)	39.5 (1.1)	33 (0.3)	55.5 (1.2)	63.2 (1.5)	35.8 (0.9)	57.1 (1.6)	72.2 (1.3)	28.9 (1.2)	60.3 (0.9)	43.9 (1.3)	53.2 (1.3)	41.5 (0.8)	30.7 (0.9)	36.9 (1.3)	53.3 (1.7)
P4	53.4 (1.4)	58.5 (1.2)	69.1 (1.3)	62.6 (0.6)	66.5 (0.8)	27.1 (1.2)	76.3 (0.9)	34.5 (0.8)	41.2 (1.2)	31.6 (0.8)	52.4 (1.4)	66.7 (0.7)	34.5 (0.8)	56.4 (1.1)	66.9 (1.4)	32.3 (1.6)	60.3 (1.3)	46.5 (0.9)	50.9 (1.3)	42.5 (1.2)	32.1 (1.4)	37.8 (0.7)	54.6 (1.1)
P5	52.1 (1.6)	54.8 (1.7)	70 (1.7)	66.2 (1.5)	66.4 (1.3)	28.2 (1.5)	79 (1.2)	35.5 (1.0)	39.9 (1.5)	32.8 (1.2)	50.1 (1.2)	64.2 (1.5)	37.4 (0.8)	59.3 (0.9)	68.2 (1.6)	30.4 (1.0)	62.1 (0.9)	45.3 (1.3)	50 (1.5)	42 (1.6)	31.5 (1.0)	39.5 (0.9)	55.3 (1.3)
P6	50.7 (1.4)	57.7 (1.4)	65.9 (1.7)	65.7 (0.6)	62.3 (1.0)	25.2 (1.4)	74.7 (1.4)	37.2 (1.6)	42.1 (1.4)	34.7 (1.3)	53.8 (1.7)	62 (1.3)	36.5 (1.0)	62 (1.4)	74.1 (1.2)	29.6 (0.8)	59.5 (0.9)	44 (1.3)	52 (1.5)	40.2 (0.9)	29.5 (0.7)	36 (1.5)	52 (1.4)
P7	53.2 (1.3)	53.5 (0.9)	66.9 (1.4)	68.6 (0.8)	63.7 (1.1)	29.4 (1.0)	73.9 (1.8)	33.7 (1.3)	41.2 (1.4)	37.3 (1.4)	57.5 (1.1)	66 (1.4)	39.6 (0.7)	58.7 (1.7)	66.7 (1.2)	34 (1.4)	59.8 (1.3)	47.4 (0.7)	53.2 (1.4)	44.2 (1.6)	34.3 (1.5)	37.1 (1.3)	52.8 (1.7)
P8	52.5 (0.8)	56 (1.2)	70.2 (1.4)	66.5 (0.8)	61.6 (1.4)	27 (1.3)	76.1 (1.4)	35.6 (1.2)	39.6 (0.9)	31 (1.6)	52.6 (0.9)	64.9 (2.3)	37.6 (0.7)	59.8 (1.3)	71.3 (1.1)	31.9 (1.4)	63.7 (1.3)	45.3 (0.9)	50.2 (1.0)	43.7 (1.4)	33.5 (1.1)	40 (1.5)	55.4 (1.9)
P8a	52.4 (1.4)	56.5 (0.9)	69.9 (1.4)	66.1 (1.3)	61 (1.2)	26 (1.3)	75.4 (1.2)	35.3 (1.3)	38.8 (0.9)	30.6 (0.9)	51.3 (1.1)	64.7 (1.2)	36.7 (1.6)	58.7 (1.6)	71 (1.3)	31.2 (1.2)	62.7 (1.5)	45.1 (1.3)	49.7 (0.9)	42.7 (1.4)	32.2 (1.1)	49.4 (0.8)	54 (1.2)
MEAN	52.4	55.6	68.1	65.6	63.4	27.2	75.7	35.7	40.8	33.3	54.1	64.2	36.5	59.0	69.5	31.3	60.6	45.1	51.4	42.1	31.7	37.7	53.7
SD	1.16	1.76	1.61	1.86	2.05	1.53	1.78	1.15	1.00	2.28	2.40	1.63	1.88	1.82	2.82	1.69	1.55	1.55	1.43	1.47	1.72	1.47	1.29
L1	44.3 (1.2)	48.3 (1.3)	62.3 (1.0)	46.2 (1.3)	61.1 (1.4)	41.6 (1.0)	64.7 (1.2)	33 (0.3)	34.2 (1.6)	73.2 (1.2)	43.7 (1.4)	54.5 (1.2)	53 (1.5)	43.7 (1.4)	60.1 (1.9)	65.3 (1.4)	55.6 (0.8)	75.3 (0.8)	39.9 (1.5)	78.4 (0.9)	75 (1.7)	32.9 (1.5)	48.5 (1.7)
L2	47.8 (1.8)	45.9 (1.6)	57 (1.7)	48.5 (1.7)	55.1 (1.5)	43.2 (1.5)	62.7 (0.8)	31 (1.6)	30.7 (0.9)	74.2 (1.4)	48.7 (1.5)	53.1 (1.2)	55.5 (1.2)	46.2 (1.3)	55.2 (0.9)	64.2 (1.4)	50.3 (0.9)	76.5 (0.7)	37.5 (0.9)	79.9 (1.5)	76.4 (0.8)	29.5 (0.7)	45.1 (1.4)
L3	46.2 (1.3)	48.8 (1.4)	59.7 (1.4)	45.8 (2.2)	59.9 (1.6)	39.9 (1.5)	64.2 (1.4)	32.7 (0.9)	32.9 (1.5)	71.7 (1.3)	45.6 (1.3)	49.6 (0.6)	54.4 (1.2)	47.1 (1.5)	57.6 (1.1)	62.7 (0.8)	54 (1.4)	73 (1.4)	41.3 (0.7)	77.7 (0.8)	78.2 (1.5)	31.7 (1.0)	47.9 (1.1)
L4	47.2 (1.0)	46.8 (1.3)	63.5 (1.5)	47.2 (1.4)	58.5 (1.2)	41.9 (1.5)	61.8 (1.4)	33.6 (1.1)	30.8 (0.9)	74.4 (1.2)	43.9 (1.3)	54.7 (0.9)	52.2 (1.6)	43.9 (1.3)	60.3 (0.9)	65.2 (1.3)	56.2 (1.5)	75.3 (0.8)	39.2 (1.0)	81.2 (1.1)	76.8 (1.2)	34.2 (1.6)	49.6 (0.6)
L5	45.3 (1.3)	46.4 (0.8)	55.4 (1.9)	49.3 (1.3)	54.2 (0.4)	40.7 (1.6)	62.8 (1.2)	31.2 (1.2)	32 (1.4)	72.7 (1.0)	46.8 (1.3)	51 (1.3)	54.6 (1.5)	50 (1.5)	59 (1.5)	62.8 (1.2)	50.9 (1.6)	73.9 (1.8)	40.8 (2.0)	79.7 (1.5)	75 (1.6)	28.7 (1.4)	43.5 (1.4)
L6	48.8 (1.4)	50.4 (0.9)	57.6 (1.1)	45.8 (2.2)	60.7 (1.2)	44.5 (0.8)	65.9 (1.7)	35.4 (1.2)	34.2 (1.3)	76.8 (1.4)	49.7 (0.9)	51.5 (0.9)	55.9 (1.6)	48 (1.3)	56.1 (1.4)	63.5 (1.5)	50.9 (1.3)	76.1 (1.4)	38 (2.2)	78.6 (0.9)	78.2 (1.5)	29.8 (1.7)	46.2 (1.3)
L7	46.5 (0.9)	49.5 (0.7)	62.6 (1.2)	50.1 (1.6)	58.2 (1.2)	42.9 (1.9)	61.3 (1.1)	33.1 (1.7)	34.7 (1.3)	74.6 (0.9)	44.6 (1.1)	57.8 (1.7)	50.6 (0.9)	44.4 (1.7)	53 (1.5)	60.6 (1.0)	54.8 (1.7)	77.5 (1.0)	42.7 (1.4)	75.9 (1.4)	80.6 (0.9)	32.8 (1.2)	48.5 (1.7)
L8	47.6 (0.9)	46.4 (0.8)	61 (1.2)	47.7 (1.4)	59.2 (1.5)	40.5 (0.8)	65.1 (1.5)	30.8 (0.9)	31 (1.1)	71.7 (1.2)	48.1 (1.2)	55 (1.5)	53 (1.4)	45.1 (1.3)	60.1 (1.5)	65.5 (0.8)	53.8 (1.7)	75.3 (1.2)	39.4 (1.6)	81.4 (0.9)	75 (1.7)	31.8 (0.9)	47.9 (1.7)
L8a	47.3 (1.4)	45.9 (1.6)	60.2 (1.2)	47.3 (1.4)	59.4 (1.3)	40 (1.5)	65.7 (0.6)	29.8 (1.1)	30.7 (0.9)	71.2 (1.8)	47.2 (1.4)	53.9 (1.0)	52.8 (1.3)	44.6 (1.1)	59.4 (1.3)	65.1 (1.5)	52.7 (0.9)	75 (1.7)	38.2 (1.4)	80.4 (1.2)	74.3 (1.3)	31.3 (0.7)	47.7 (1.4)
MEAN	46.7	47.8	59.9	47.6	58.4	41.9	63.6	32.6	32.6	73.7	46.4	53.4	53.7	46.1	57.7	63.7	53.3	75.4	39.9	79.1	76.9	31.4	47.1
SD	1.45	1.67	2.96	1.63	2.51	1.56	1.65	1.56	1.66	1.71	2.29	2.64	1.78	2.21	2.70	1.68	2.31	1.42	1.72	1.84	2.01	1.92	2.03
E1	41.8 (0.9)	42.7 (1.4)	43.3 (0.8)	39.5 (0.8)	48.9 (1.3)	17.3 (0.9)	42.6 (0.9)	28.9 (1.2)	28.3 (1.1)	26 (1.4)	25.4 (1.4)	36.4 (0.8)	28.3 (1.1)	75.9 (1.4)	38.1 (1.2)	21.7 (1.3)	38.6 (0.7)	36.7 (1.6)	64 (1.4)	31.5 (0.8)	24.9 (1.5)	19.1 (1.3)	34.4 (1.1)
E2	40.4 (1.2)	40.2 (0.9)	40.8 (1.3)	36.9 (1.3)	46.9 (1.2)	15.3 (1.3)	41.4 (0.9)	27.7 (0.8)	25.7 (1.4)	23.7 (1.5)	26.8 (0.4)	31.2 (1.2)	24.8 (1.6)	74.1 (1.2)	34.8 (0.9)	18.7 (1.5)	36.7 (0.8)	33.8 (1.3)	62.1 (0.9)	34.2 (1.6)	21.7 (1.3)	16.9 (1.1)	31.5 (1.0)
E3	39.5 (0.8)	40.9 (1.3)	42.8 (1.5)	38.2 (1.4)	45.1 (1.3)	16.2 (1.3)	44 (1.3)	29.6 (1.0)	27.5 (1.1)	27.1 (1.2)	23.9 (1.1)	35.2 (1.2)	23.9 (1.1)	78.5 (1.0)	35.6 (1.2)	20 (1.8)	37.8 (1.6)	35 (1.4)	65.2 (1.3)	32.9 (1.5)	22.5 (0.8)	18 (1.6)	32.5 (0.8)
E4	40.1 (1.4)	42.9 (1.9)	44.4 (1.7)	40.6 (0.8)	48.1 (1.2)	14.6 (0.9)	41.1 (1.6)	27.1 (1.2)	26.5 (1.2)	25.6 (0.7)	27.7 (0.8)	34.5 (0.9)	27 (1.6)	79.2 (1.6)	34.5 (0.8)	17.9 (1.3)	40.7 (1.0)	33 (0.3)	62.4 (0.9)	34.7 (1.3)	24.9 (1.5)	20 (2.0)	35.1 (1.5)
E5	41.3 (0.8)	41.1 (1.5)	42.4 (1.7)	39.2 (1.6)	45.4 (1.2)	17.5 (0.7)	42.4 (1.7)	28.8 (1.2)	25 (1.6)	24.5 (0.7)	25.2 (1.4)	36.4 (0.8)	28.7 (1.1)	76.1 (1.4)	37.4 (0.8)	20.9 (1.4)	38 (2.2)	36 (1.5)	64.9 (2.3)	32.2 (1.1)	23.1 (1.5)	18.8 (1.4)	33.1 (1.7)

E6	37.9 (1.2)	39.7 (1.3)	43.3 (0.8)	36.9 (1.3)	47.4 (0.7)	18.9 (1.6)	45.5 (0.9)	30.2 (1.4)	28.8 (1.2)	29.2 (0.9)	29.1 (1.7)	30.8 (0.9)	23.7 (1.5)	78.1 (1.3)	40.5 (0.8)	22.9 (1.7)	37.7 (2.0)	38 (1.5)	66.9 (1.4)	36.3 (1.2)	26.3 (1.3)	18.1 (1.5)	33 (1.1)
E7	38.8 (0.9)	38.8 (0.9)	39.7 (1.3)	34.3 (1.5)	47.5 (0.7)	16.7 (1.6)	44.5 (0.8)	27.3 (1.7)	26.3 (1.3)	26.2 (1.6)	23.9 (1.1)	29.8 (1.1)	25.6 (0.7)	74.9 (1.3)	37 (1.6)	17.9 (1.5)	35.1 (1.5)	35.4 (1.2)	62.1 (1.4)	33.5 (1.1)	21.5 (0.7)	16.2 (1.7)	31.8 (1.4)
E8	40.1 (1.4)	41.7 (1.4)	46.6 (1.1)	41.4 (0.9)	42.8 (1.5)	17.1 (1.0)	41.8 (0.9)	28.4 (1.0)	28 (1.3)	23.5 (1.2)	26.8 (1.3)	34.3 (1.5)	26.8 (1.3)	79 (1.5)	33.4 (0.7)	19.6 (1.7)	40.2 (1.4)	33.5 (1.4)	63.5 (1.5)	34.5 (0.8)	24.9 (1.5)	21 (1.6)	36.6 (1.6)
E8a	40.4 (0.7)	41.5 (0.8)	45.6 (1.3)	40.4 (0.7)	42.4 (1.7)	16.2 (1.3)	41.2 (1.4)	29 (1.5)	27.6 (1.0)	22.2 (1.5)	26 (1.4)	34.4 (1.1)	26.3 (1.3)	78.3 (0.8)	33.2 (1.2)	18.6 (1.5)	39 (1.3)	33.2 (1.2)	62.8 (1.8)	33.1 (1.7)	23.7 (1.5)	20.3 (0.8)	36.1 (1.4)
MEAN	40.0	41.0	42.9	38.4	46.5	16.7	42.9	28.5	27.0	25.7	26.1	33.6	26.1	77.0	36.4	20.0	38.1	35.2	63.9	33.7	23.7	18.5	33.5
SD	1.27	1.42	2.10	2.30	1.97	1.34	1.58	1.10	1.34	1.88	1.83	2.61	1.91	1.99	2.29	1.80	1.82	1.71	1.72	1.53	1.76	1.57	1.74

H Healthy volunteers, Pv Pemphigus vulgaris patients, P Psoriasis patients, L Leishmania patients, E Eczema patients, a spiked samples
The values given in parenthesis represents standard deviation

Table 3: Amino acids and polyamines contents ($\mu\text{g/g}$) in skin samples of healthy volunteers, pemphigus vulgaris, psoriasis, leishmania and eczema patients.

	H (n=10)	PV (n=8)	P (n=8)	L (n=8)	E (n=8)
Gly	65-70.2 67.8 (1.78)	58.5-61.9 59.8 (1.29)	50.7-54.1 52.4 (1.16)	44.3-48.8 46.7 (1.45)	37.9-41.8 40 (1.27)
Ala	65.1-70.4 67.9 (1.86)	57.7-62.4 59.9 (1.47)	53.5-58.5 55.6 (1.76)	45.9-50.4 47.8 (1.67)	38.8-42.9 41 (1.42)
Put	42.4-53.4 47.8 (3.45)	59.9-65.7 63.6 (1.97)	65.9-70.2 68.1 (1.61)	55.4-63.5 59.9 (2.96)	39.7-46.6 42.9 (2.1)
Ser	91.3-97.7 94.3 (2.03)	76.6-80.8 78.6 (1.38)	62.6-68.6 65.6 (1.86)	45.8-50.1 47.6 (1.63)	34.3-41.4 38.4 (2.3)
Pro	75.9-83 78.2 (2.22)	66.1-73.9 70.1 (2.84)	61.4-66.5 63.4 (2.05)	54.2-61.1 58.4 (2.51)	42.8-48.9 46.5 (1.97)
Val	18.8-23.2 21.3 (1.62)	31.7-36.1 33.9 (1.35)	25.2-29.4 27.2 (1.53)	39.9-44.5 41.9 (1.56)	14.6-18.9 16.7 (1.34)
Thr	31.9-37.3 35 (2.14)	49.7-55.2 52 (1.79)	73.9-79 75.7 (1.78)	61.3-65.9 63.6 (1.65)	41.1-45.5 42.9 (1.58)
Leu	51.2-56.5 53.6 (1.93)	41.5-45.6 43.2 (1.26)	33.7-37.2 35.7 (1.15)	30.8-35.4 32.6 (1.56)	27.1-30.2 28.5 (1.1)
Ile	50.5-56.9 53.7 (1.98)	45.2-49.1 47 (1.34)	39.5-42.1 40.8 (1.0)	30.7-34.7 32.6 (1.66)	25-28.8 27 (1.34)
Cys	29.2-46.8 42.8 (2.24)	55.7-61.5 57.8 (2)	30.7-37.3 33.3 (2.28)	71.7-76.8 73.7 (1.71)	23.5-29.2 25.7 (1.88)
Asn	28.1-35.3 32.5 (2.74)	64.5-71.1 67.4 (2.15)	50.1-57.5 54.1 (2.4)	43.7-49.7 46.4 (2.29)	23.9-29.1 26.1 (1.83)
Asp	19-27 23.1 (3.23)	39.9-47.7 43.7 (2.67)	62-66.7 64.2 (1.63)	49.6-57.8 53.4 (2.64)	29.8-36.4 33.6 (2.61)
Lys	37.4-46.8 43.2 (2.78)	59.7-65.4 61.9 (1.91)	33.6-39.6 36.5 (1.88)	50.6-55.9 53.7 (1.78)	23.7-28.7 26.1 (1.91)
Gln	23.4-33.8 28.9 (3.5)	18.4-24.7 21.2 (2.08)	56.4-62 59 (1.82)	47.7-50 46.1 (2.21)	74-79.2 77 (1.99)
Glu	22-29.9 26.1 (2.87)	45.1-51.7 47.7 (2.15)	66.7-74.1 69.5 (2.82)	53-60.3 57.7 (2.7)	33.4-40.5 36.4 (2.29)
Met	35.2-41.7 38.4 (2.4)	51.2-56.4 53.2 (1.74)	28.9-34 31.3 (1.69)	60.6-65.5 63.7 (1.68)	17.9-22.9 20 (1.8)
Spd	37.5-46.5 42.4 (2.85)	53.7-59 56.6 (1.59)	58.8-63.7 60.6 (1.55)	50.3-56.2 53.3 (2.31)	35.1-40.7 38.1 (1.82)
His	52.9-61.2 57.2 (2.49)	65.9-69.9 67.6 (1.45)	42.6-47.4 45.1 (1.55)	73-77.5 75.4 (1.42)	33-38 35.2 (1.71)
Phe	23-31.7	19.5-25.5	49.5-53.2	37.5-42.7	62.1-66.9

	28.4 (3.01)	22 (1.95)	51.4 (1.43)	39.9 (1.72)	63.9 (1.72)
Arg	43.3-53	58.8-62.9	40.2-44.2	75.9-81.4	31.5-36.3
	47.8 (2.81)	61 (1.51)	42.1 (1.47)	79.1 (1.84)	33.7 (1.53)
Tyr	35.1-44.6	46.8-52.6	29.5-33.5	75-80.6	21.5-26.3
	40.5 (3.18)	50.5 (1.89)	31.7 (1.72)	76.9 (2.01)	23.7 (1.76)
Trp	18.7-26.1	31.8-35.8	36-40	28.7-34.2	16.9-21
	22.2 (2.51)	34.3 (1.29)	37.7 (1.74)	31.4 (1.92)	18.5 (1.57)
Spm	33.4-41.5	47.2-51.8	52-55.4	43.5-49.6	31.5-36.6
	37.4 (2.48)	50.1 (1.58)	53.7 (1.29)	47.1 (2.03)	33.5 (1.74)

H Healthy volunteers, Pv Pemphigus vulgaris patients, P Psoriasis patients, L Leishmania patients, E Eczema patients, n Number of samples analysed

Table 4: Quantitative analysis of amino acids and polyamines in skin samples with range (µg/g), mean values (standard deviation).

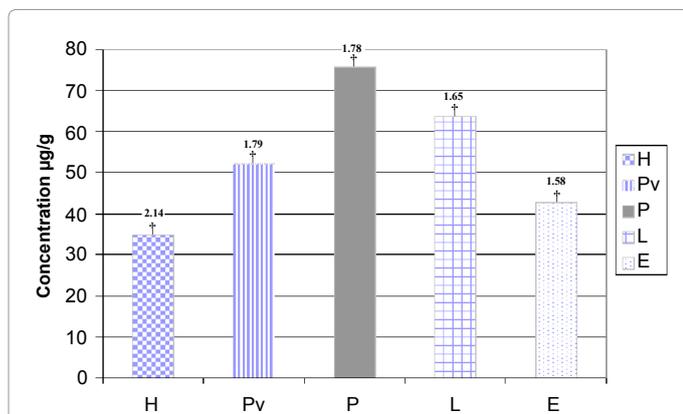


Figure 3: The variation in the average concentration µg/g with standard deviation for threonine in H=healthy volunteers (n=10), Pv=pemphigus vulgaris (n=8), P=psoriasis (n=8), L=leishmaniasis (n=8) and E=eczema (n=8) concentration as table 3.

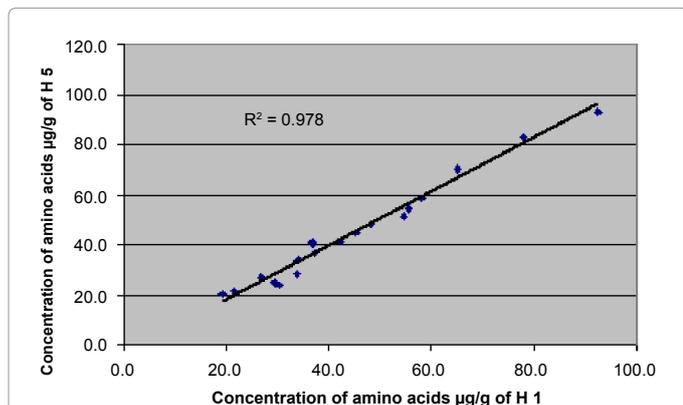


Figure 4a: Coefficient of determination (r^2) of 20 amino acids and 03 polyamines for healthy volunteers 1 and 5. The concentration of amino acids and polyamines as table 2. GC conditions as fig 1.

Table 4. A sample from each of the group H, Pv, P, L and E was also analyzed by standard addition technique and the results obtained agreed with direct calibration with recovery within 95-102 % with RSDs within 0.94-7.78 %.

To understand these results, the amino acids were grouped by chemical features and differences between the skin samples were analyzed.

The aliphatic amino acids Gly, Ala, Val, Leu and Ile present in significant amount in all the skin samples were examined. Gly, Ala, Leu

and Ile were higher in healthy volunteers and minimum in eczema with the decreasing order: H > Pv > P > L > E. Val was observed higher in L with following decreasing order: L > Pv > P > H > E. Among hydroxy amino acids Thr and Ser, the highest concentration of Thr was obtained in P with decreasing order: P > L > Pv > E > H and Ser was highest in H with a sequence H > Pv > P > L > E. Among sulphur containing amino acids Met, and Cys the highest concentration for both was in L with decreasing order L > Pv > H > P > E. Among acidic amino acids Glu and Asp and amide Asn the highest concentration of Glu and Asp was observed in P with decreasing order: P > L > Pv > E > H. Where as in the case of Asn the order was somewhat different Pv > P > L > H > E. The other amino acids such as aromatic (Phe) and basic (Lys) were also examined. Phe indicated the concentration of amino acids in the sequence E > P > L > H > Pv and Lys in Pv > L > H > P > E while polyamines Put, Spd, Spm indicated the concentration in the following decreasing order P > Pv > L > H > E (Figure 3).

The concentration of amino acids and polyamines were observed in the range of µg/g H 18.7-97.7, Pv 18.4-80.8, P 25.2-79, L 28.7-81.4 and E 14.6-79.2. The average results (n=10) for H indicated following decreasing order of concentration: Ser > Pro > Ala > Gly > His > Ile > Leu > Put > Arg > Lys > Cys > Spd > Tyr > Met > Spm > Thr > Asn > Gln > Phe > Glu > Asp > Trp > Val. The concentration order was somewhat different in the affected skin samples. Pv indicated Ser > Pro > His > Asn > Put > Lys > Arg > Ala > Gly > Cys > Spd > Met > Thr > Tyr > Spm > Glu > Ile > Asp > Leu > Trp > Val > Phe > Gln. P indicated Thr > Glu > Put > Ser > Asp > Pro > Spd > Gln > Ala > Asn > Spm > Gly > Phe > His > Arg > Ile > Trp > Lys > Leu > Cys > Tyr > Met > Val. L showed the following order Arg > Tyr > His > Cys > Met > Thr > Pro > Glu > Lys > Asp > Spd > Ala > Ser > Spm > Gly > Asn > Gln > Val > Phe > Ile > Leu > Trp. Finally E patients indicated amino acids concentration in the decreasing order: Gln > Phe > Pro > Put > Thr > Ala > Gly > Ser > Spd > Glu > His > Arg > Asp > Spm > Leu > Ile > Asn > Lys > Cys > Tyr > Met > Trp > Val.

The coefficient of determination (r^2) among the healthy volunteers for 23 compounds concentration µg/g were examined and r^2 were observed within 0.9659-0.9878, but when the average concentration values of healthy volunteers (n=10) on the x-axis were plotted against the affected skin samples of Pv, P, L and E on Y-axis (n=8), the values of r^2 were obtained 0.5162, 0.0364, 0.0045 and 0.0094 respectively. The results indicated that the amino acids from healthy volunteers agreed with each other, but they differed significantly from the affected skin samples from Pv, P, L and E (Figure 4 a, b).

Similarly the results of analysis of patients with the same disease agreed with each other with r^2 within 0.9621-0.9938, but differed from different diseases like Pv-P, Pv-L, Pv-E, P-L, P-E, and L-E with r^2 0.0265, 0.1879, 0.0943, 0.0005, 0.3489 and 0.0067 respectively and supports the

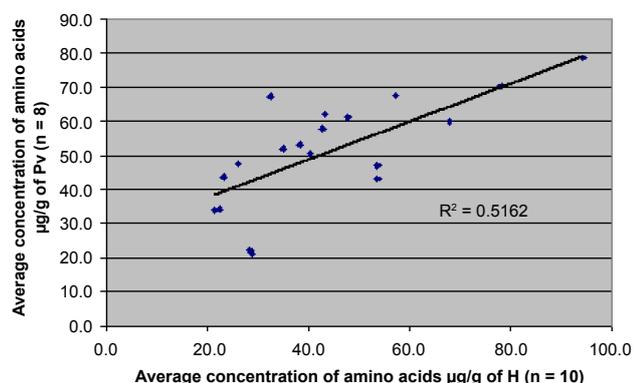


Figure 4b: Coefficient of determination (r^2) of average concentration of 20 amino acids and 03 polyamines for healthy volunteers ($n = 10$) against the average concentration of amino acids and polyamines of Pv ($n = 8$) as in table 4. GC conditions as figure 1.

hypothesis that the diseases Pv, P, L and E alter the protein molecules differently in the keratinocytes of the skin.

Conclusions

An analytical procedure has been developed for GC determination of 20 amino acids, 5 polyamines and 3 biological active compounds using double derivatization with FAA and IBCF with improved sensitivity. The method is applied for the analysis of amino acids and polyamines from acid hydrolyzed human skin samples from the patients of pemphigus vulgaris, Psoriasis, leishmaniac, eczema and healthy volunteers. A variation in amino acids contents among the patients and healthy volunteers is noted.

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