Determination of Alanine, Arginine, and Tyrosine in Black, Oolong, Green and White tea by Derivation with Ortho-Phthalaldehyde Using HPLC-FLD

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☐ ABSTRACT ☐

A simple gradient HPLC method utilise a fluorescence detector (FLD) was developed for highly sensitive, rapid and reliable quantitative determination of Alanine, Arginine, and Tyrosine found mainly in commercial tea types of black, oolong, green, and white.

The method depends on the derivation of amino acids, with a mixture of O-Phthalaldehyde and 3-Mercaptopropionic acid reagents.

The chromatographic separation was achieved using a reversed-phase Agilent Zorbax Exclipe RP18 (C18), 250 mm × 4.6 mm, 5 μm column depending on the gradient HPLC method employs in two solvents A and B as mobile phase. Solvent A contains a mixture of tetrahydrofuran: acetate buffer pH 7.6 (3:97, v/v), and solvent B contains a mixture of acetate buffer pH 7.6 : acetonitrile : methanol (1:2:2,v/v/v). The flow rate of mobile phase was 1.5 mL/min, The FL detector was set at excitation wavelength (λ_{Ex}) of 340 nm and emission wavelength (λ_{Em}) of 450 nm. The calculated detection limit LOD for amino acids were very low, in the range of 1.214 – 5.592 nmol/L, with very low limit of quantification LOQ range of 3.680 – 16.945 nmol/L and RSD range   1.05 – 2.87%.

The developed RP-HPLC method was validated with respect to accuracy, precision and was shown advantageous over the simpler methodology with highly precision, accuracy, sensitive, and negligible interference of natural present in tea infusion. The proposed method was successfully applied for the separation and determination of Alanine, Arginine, and Tyrosine in eleven tea samples of black, oolong, green and white found in the local market.

Keywords; Simultaneous determination; amino acids; Alanine; Arginine; Tyrosine; O-Phthalaldehyde; 3Mercaptopropionic acid; Tea infusions.

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تحديد الألانين والأرجنين والتيروزين في عينات من الشاي الأسود والأولنج والأخضر والأبيض بالتحليل الكروماتوغرافي السائل عالي الأداء المزود بكاشف الفلورة عن طريق الاشتقاق بأورتو فتال ألدهيد

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رنا قازان**

□ ملخّص □

طورت طريقة تحليلية كروماتوغرافية بسيطة وسريعة وذات حساسية عالية باستخدام كاشف الفلورة من أجل التحديد الكمي للألانين والأرجنين والتيروزين المتواجدين بشكل رئيسي في أنواع الشاي المعروفة كالشاي الأسود والأولنج والأخضر والأبيض.

تعتمد الطريقة على اشتقاق الحموض الأمينية بمزيج من كاشفي الاشتقاق أورتو فتال ألدهيد Ortho– Phthalaldehyde (OPA) وَ 3 مركبتو بروبيونيك أسيد 3-MPA) acid Mercaptopropinic–3)، ومن ثم يتم الفصل على العمود الكروماتوغرافي باستخدام الطور العكوس RP C18، ذو الأبعاد 250 mm × 4.6 mm, (5 µm)، بالاعتماد على التدرج في مذيبات الطور المتحرك والذي يتكون من مذيبين A و B، يتكون المذيب A من مزيج من تتراهيدروفوران، واقي خلات pH:7.6 بنسب حجمية (97:3، v/v)، والمذيب B من واقي خلات pH:7.6، أسيتونتريل، ميثانول بنسب حجمية (2:2:1 ،v/v/v)، أجريت الدراسة عند معدل سرعة تدفق للطور المتحرك 1.5 mL/min، وباستخدام كاشف الفلورة عند طول موجة الإثارة λEx = 340 nm، وطول موجة الإصدار λEm = 450 nm، كما تراوح حد الكشف النوعي للحموض الأمينية المحددة بين nmol/L (5.592 – 1.214) وحد التحديد الكمي بين nmol/L (16.945 – 3.680) بانحراف معياري نسبي مئوي تراوح بين (2.87 – 1.05)%.

تمتاز الطريقة المطورة بالدقة والصحة والحساسية واستخدمت للفصل والتحديد المتزامن لكلٍ من الألانين والأرجنين والتيروزين في إحدى عشرة عينة من عينات محاليل الشاي الأسود والأخضر والأولنج والأبيض من دون حدوث أي تداخل مع المواد الأخرى التي توجد طبيعياً في محاليل الشاي.

الكلمات المفتاحية: التحديد المتزامن، حموض أمينية، ألانين، أرجنين، تيروزين، أورتوفثال ألدهيد، 3-مركبتو بروبيونيك أسيد، محاليل الشاي.

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Introduction.

Amino acids co-exist in biological and food matrices and participate in several transformation processes [1]. Amino acids play a central role such as building blocks of proteins and as intermediates in the metabolism. They represent an important part of the human body such as proteins found in muscles, tendons, organs, glands, nails and hair, and promote growth, repair and maintenance of the cells. Amino acids microbial catabolism produces key flavour compounds in foods such as cheese, wine, honey and other fermented foodstuff [2]

In tea (*Camellia Sinensis*), there are many amino acids with different levels and profiles according to the classification of manufacturing process; e.g. green and white tea are unfermented, oolong tea is semi fermented, whereas black tea is fully fermented. Therefore, identifying the content of amino acids and profile in tea are critical and find practical application according to the tea type in order, to standardize both of tea quality and grade [3].

Traditionally amino acids assayed in tea by ninhydrin method [4], although liquid chromatography [5,6] is the most commonly used considering the fact that most of the amino acid materials lack of a strong chromophore or fluorophore.

Conventional chromatographic methods have being far from straightforward utilizing ultraviolet, visible or fluorescence detectors. Traditional detection methods involve chemical derivation with functional groups to yield highly detectable amino acids level either prior to or within the HPLC system.

Among the most commonly used derivation reagents for HPLC analysis of amino acids are Ninhydrin [7,8], Dansyl chloride [9,10], 9-Fluorenylmethyl chloroformate (FMOC-Cl) [11,12], Phenylisothiocyanate (PITC) [13,14], and O-Phthalaldehyde (OPA) [15,16].

As far as the present work concerns, the OPA material itself is non fluorescent that binds rapidly with primary amino acids at room temperature to form highly fluorescent isoindoles that used for pre- and post-column derivation. However, the disadvantages of utilizing this method is the lack of OPA activity with secondary amino acids [15,16] as well as the formation of unstable OPA derivatives. Therefore, a stabilizing reagents such as mercaptan cofactor (contain SH-group) are always required that is incorporated as a part of the final derivative product, Scheme 1 [17].

The choice of mercaptan can affect derivative stability and chromatography selectivity. The most commonly SH-group utilized for OPA reaction are   2-Mercaptoethanol (2-MCE) [18,19], Ethanethiol [11], and 3- Mercaptopropionic acid [16,20,21].
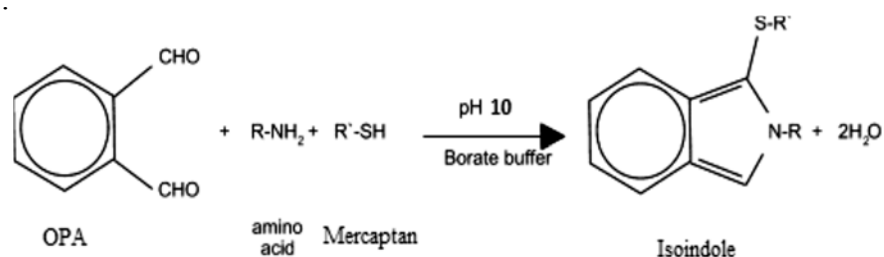
scheme 1. Formation of isoindole compound (fluorophor).

In this work, a simple method is presented for the determination of three primary amino acids usually found in tea. The detection is performed by HPLC separation of amino acids/OPA/3-MPA derivatives to form relatively stable intensely fluorescent isoindoles.

The potentiality of the described method for simultaneous quantification of the same amino acids found in trace levels in common tea type is also reported.
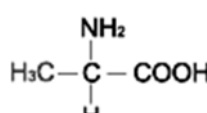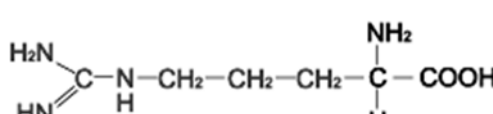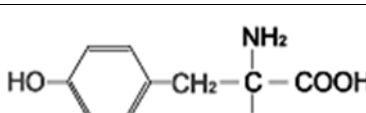
Experimental.

2.1 Chemicals and materials.

Amino Acids studied in this work are presented in Table 1: DL Alanine (Ala), L-Arginine HCl (Arg), and D-Tyrosine (Tyr), of high purity (≥ 98%) obtained from Sigma-Aldrich (U.S.A) and used for standard preparations. HPLC solvents of methanol, Tetrahydrofuran and acetonitrile were of HPLC grade and purchased from Merck company (Germany). Reagent grade of sodium acetate trihydrate, sodium borate, hydrochloric acid, sodium hydroxide, and glacial acetic acid obtained from Scharlau (Spain).

Derivation reagents of Ortho-Phtalaldehyde (OPA) (98%) and 3-Mercaptopropionic acid (3-MPA) (99%) were obtained from ALFA AeSar (Karlsruhe, Germany). All chemicals were used as received without further purification. The deionized water used for the preparation of standard solutions, buffers, dilution and eluents was conductivity < 1μs/cm.

All mobile phases and prepared solution were filtered prior to use through a 0.45 μm nylon filter (Whatman, Hillsboro, OR). Solutions to be injected through the HPLC system were filtered through 0.45-μm MDI Nylon syringe filters (Advanced Microdevices Pvt. Ltd., Ambala Cantt, India).

Table 1: Name, Three-letter abbreviation, Molecular Formula, and Molecular Weight.

Name	Three-letter abbreviation	Structural formula	Molecular Formula	Molecular Weight (g/mol)
Alanine	ALa		$C_3H_7NO_2$	89.09
Arginine	Arg		$C_6H_{14}N_4O_2$	174.20
Tyrosine	Tyr		$C_9H_{11}NO_3$	181.19

2.2 Standard preparations.

Amino acids stock solution of 1.0×10^{-3} M was prepared by dissolving a proper quantity of each individual amino acid in 0.1 M HCl and was stored frozen for further preparation.

Dilution of the stock solutions with 0.1 M HCl were performed in order to achive the appropriate standard concentrations for the calibration curve, then filtered through 0.45 μM before further uses. The HPLC peaks were generated upon injection of amino acids to the final concentration of 55.0 μM.

3-Mercaptopropionic acid (3-MPA) stock solution of 1.0×10^{-3} M concentration was prepared weekly by proper dilution with methanol. Borate buffer was prepared by adjusting the pH of 0.4 M sodium borate solution to the value of 10 using NaOH.

Ortho- Phthalaldehyde (OPA) stock solution was prepared weekly at a concentration of 1.0×10^{-3} M in borate buffer. Stock solutions of 3-MPA and OPA were stored at   4 °C in a refrigerator.

2.3 Derivation procedure.

The derivation reagent was prepared daily by mixing 15 mL OPA and 5 mL 3-MPA. A volume of 5 mL of this reagents mixture was orderly added to 10 mL sample (or standard solution), allowed to react for 5 min at ambient temperature for pre-HPLC column derivation, filtered through 0.45 µm filter and then an aliquot (20 µL volume) of this solution was immediately injected through HPLC system. The reagent blanks free from amino acids were also treated in the same way.

2.4 Tea samples preparation.

Eleven commercial tea samples purchased from retail local markets were investigated in this study. These samples include five black teas types from different origins i.e. one Silane origin (Sri Lanka) (Sam 1), one Indian origin (Sam 2), and other three different Chinese grades (A,B,C) (Sam 3 to 5). The other tea type were of two green tea samples (Sam 6 & 7), two oolong teas (Sam 8 & 9), and two white teas (Sam 10 & 11), each type is differed in the cultivating origins of Sri Lanka and China, respectively.

Constant 2.5g net weight of each individual tea sample was grounded into homogenous powder using a grinder (Joyoung Co., Jinan, Shandong, China), and stored in the desiccators at room temperature before infusion. Tea powder of 0.30 g was extracted by 20mL distilled water at 80 °C for 20 min (staring well during the incubation) and then cooled to room temperature.

Subsequently, the infusion was collected by centrifuging the solid matter in a centrifuge at 12,000 rpm for 10 min, make the volume again up to 20 mL with Borate buffer, and filtered through a 0.45 µm nylon filter membrane. A 1 mL volume of the obtained filtrate is diluted with water to 10 mL and used as infusion tea sample for pre-column reaction with 5 mL derivation reagents and proceed for HPLC measurement. Time line for each individual step of the process preparation from tea powder dunking to the HPLC injection was set as constant during 60 min of the total experimental time.

2.5 HPLC analysis.

An HPLC system equipped with fluorescence detector (FLD), binary pump and column temperature regulator all of Agilent Technology series 1200 were used. Chromatography *Chemstation* software (Agilent, German) was used for the instrument control and data processing.

The fluorescence detection wavelengths were set at excitation wavelength (λ_{Ex}= 340 nm), and emission wavelength (λ_{Em}= 450 nm). Sample injection volume was 20 µL. The HPLC separations were performed at 32 °C on Agilent Zorbax Exclipe XDB-C18 column (5 µm, 250 mm × 4.6 mm i.d., Agilent Technologies, U.S.A.) with a binary gradient mobile phase. The flow rate was set at 1.5 mL min^{-1}. Acetate buffer (20 mM) and (100 mM) was used for mobile phase, and we adjusted them at pH = 7.6 using (2%) of glacial acetic acid.

Solvent A was 20 mM acetate buffer with 3.0% Tetrahydrofuran and solvent B was a mixture of 100 mM acetate buffer: acetonitrile: methanol of 1:2:2 volumetric ratio. The gradient elution time was programmed as indicated in Table 2 with overall record

chromatogram time of 10 min. Between analysis, the HPLC column was pre-equilibrated with isocratic elution mode of solvent A as a mobile phase.

Table 2: Gradient program employed for the separation of amino acids.

Time (min)	Flow (ml/min)	Mobile phase A	Mobile phase B
0	1.5	100	0
0.7	1.5	94	6
8	1.5	76	24
9.5	1.5	0	100
10	1.5	100	0

Results and discussion.

3.1 Optimization of chromatographic conditions.

The derivation yield is dependent on both of the reagent concentration and their reaction time. Our pre-investigation shows that: the molar ratio of (OPA : 3-MPA / 3 : 1, and Amino acid : OPA /2:1), and reaction time of amino acids's derivation is about 5 min, using borate buffer (pH = 10, 0.4 M). [22,23].

Optimum fluorescence wavelength were determined by the variation of the excitation wavelength for the best fluorescence emission. The HPLC chromatogram of OPA, 3-MPA and a mixture of 3-MPA:OPA (1:3) solutions were examined and show no relative HPLC-peaks at the conditions applied, whereas, HPLC-peaks for 3-MPA:OPA derivation reagents with the examined amino acids exhibit narrow and well-defined peaks in relatively short elution time of 10 min.

Variation of the ratio of the optimum gradient condition of the mobile phase will prolong the amino acid retention time, with an appearance of tail or may cause peaks overlapping. The retention time obtained for each individual amino acids are listed in Table 3, and the obtained chromatogram of the three amino acids mixture is illustrated in Figure 1. These results show the potential application of pre-column derivation with OPA :3-MPA for the simultaneous detection of the indicated amino acids with no peaks overlapping.
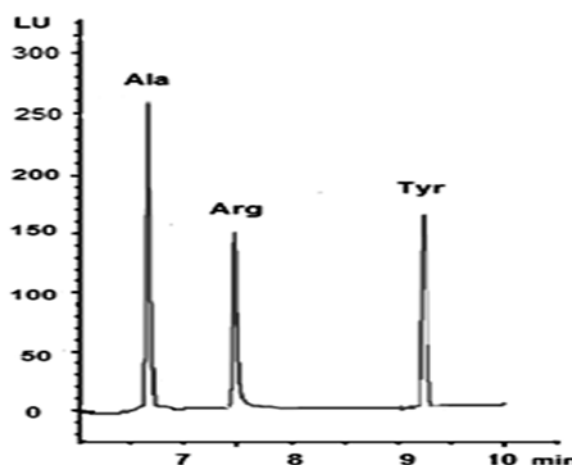

Figure 1: Chromatogram of Alanine, Arginine , and Tyrosine solution (10 µM) after pre-column derivations with OPA:3-MPA, applying the stated HPLC conditions and FL detection.

Table 3: Retention Times, Calibration Curves (y= ax + b), Correlation Coefficient (R^2),   and Linear Range.

Name	Retention time (min)	a	b	R^2	Linear Range
Alanine	6.82	36.141	- 2.3249	0.9997	0.5 – 25
Arginine	7.5	17.94	0.9702	0.9996	1 – 50
Tyrosine	9.29	16.742	25.726	0.9998	1 – 55

3.2 Method validation.

The linearity was evaluated by the construction of calibration curves (Figures 2), using the chromatographic peaks areas of the fluorescence response of five standard injections for every amino acid determined. The linearity between concentrations and peak area are all illustrated in Table 3.
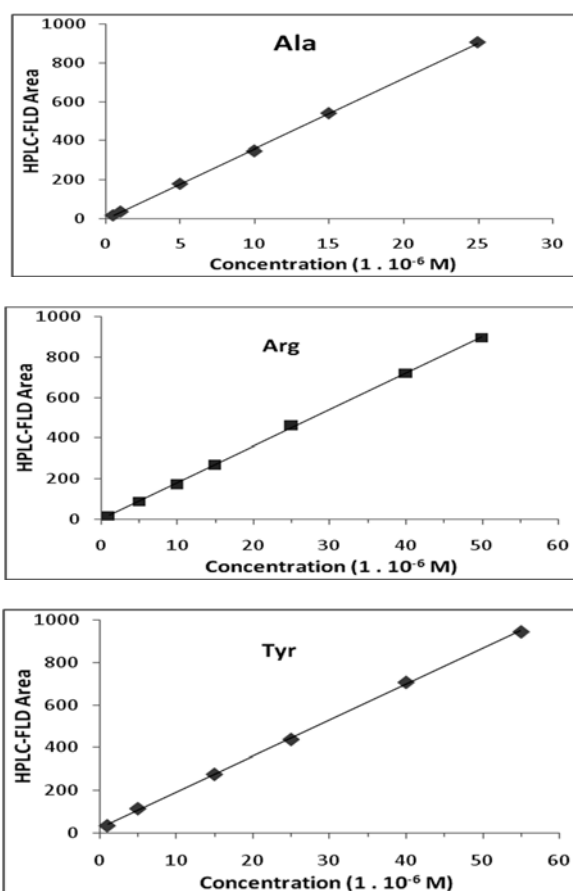

Figure 2: Calibration curves of amino acids: Alanine, Arginine, and Tyrosine

It is confirmed from the strictly linear variation of the peak response of pre-column derivation with 3-MPA : OPA towards individual amino acid at various concentrations, that the calibration curves demonstrate linearity for the amino acids studied at various concentration ranges of each individual ($R^2 \geq 0.9996$). The average recovery obtained for different amino acids was acceptable, with values ranging between (98.0 – 100.8) % for Ala, (105.0 – 108.0) % for Arg, and (93.0 – 96.0) % for Tyr. Tables 4,5 and 6.

Whatever the value of tested concentration is, a very accurate quantitative recovery is obtained with low RSD% not exceed 2.71 % for (Ala), 2.87% for (Arg) and 1.89% for (Tyr) (n = 5).

The confidence limit calculated at 95 % level of confidence, (t = 2.776).

Table 4: Accuracy and precision for the determination of Ala (n=5)

Nominal concentration μM	(μM) $\bar{x}$ Mean ± SD	RSD%	Confidence limit (μM) $\bar{x} \pm \dfrac{t \times SD}{\sqrt{n}}$	Recovery%
0.5	0.49 ± 0.0133	2.71	0.49 ± 0.0165	98.0
1	0.99 ± 0.0238	2.40	0.99 ± 0.0295	99.0
5	4.97 ± 0.1049	2.11	4.97 ± 0.1302	99.4
10	10.01 ± 0.1832	1.83	10.01 ± 0.2274	100.1
15	15.12 ± 0.2449	1.62	15.12 ± 0.3040	100.8
25	24.90 ± 0.3785	1.52	24.90 ± 0.4699	996

Table (5) Accuracy and precision for the determination of Arg (n=5)

Nominal concentration μM	(μM) $\bar{x}$ Mean ± SD	RSD%	Confidence limit (μM) $\bar{x} \pm \dfrac{t \times SD}{\sqrt{n}}$	Recovery%
1	1.06 ± 0.0304	2.87	1.06 ± 0.0377	106.0
5	5.25 ± 0.1412	2.69	5.25 ± 0.1753	105.0
10	10.54 ± 0.2635	2.50	10.54 ± 0.3271	105.4
15	15.90 ± 0.3816	2.40	15.90 ± 0.4737	106.0
25	26.75 ± 0.5912	2.21	26.75 ± 0.7340	107.0
40	42.92 ± 0.8584	2.00	42.92 ± 1.0657	107.3
50	54.00 ± 1.0476	1.94	54.00 ± 1.3006	108.0

Table 6: Accuracy and precision for the determination of Tyr (n=5)

Nominal concentration μM	(μM) $\bar{x}$ Mean ± SD	RSD%	Confidence limit (μM) $\bar{x} \pm \dfrac{t \times SD}{\sqrt{n}}$	Recovery%
1	0.93 ± 0.0176	1.89	0.93 ± 0.0218	93.0
5	4.69 ± 0.0774	1.65	4.69 ± 0.0961	93.8
15	14.20 ± 0.2002	1.41	14.20 ± 0.2485	94.6
25	23.25 ± 0.2836	1.22	23.25 ± 0.3521	93.0
40	± 0.5201 38.24	1.36	38.24 ± 6457	95.6
55	52.80 ± 0.5544	1.05	52.80 ± 0.6883	96.0

The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the formula LOD = 3.3 SD/a and LOQ = 10 SD/a, respectively, where SD is the standard deviation of five determinations for the lower concentrations studied, and b is the slope of the calibration curve [24].

In these conditions, the LOD and LOQ of the developed method were calculated and furnished in Table 7. The LOD values (n=5) are ranged from 1.214 nM for Ala , to 5.592 nM for Arg. Whereas, the LOQ values are ranged 3.680 nM for Ala, to 16.945 for Arg (n=5).

Table 7: Limit of detection (nmol/L), Limit of quantification (nmol/L).

(nmol/L)	Alanine	Arginine	Tyrosine
LOD	1.214	5.592	3.469
LOQ	3.680	16.945	10.512

3.3 Tea sample applications.

The usefulness of the developed pre-column derivation method was utilized for the determination of amino acids in eleven different tea samples of black, green, oolong and white.

The chromatogram of Sam.1 of black tea after derivations with 3-MPA: OPA is illustrated in Figure 3. This illustration shows the variation of the peak height after spiking the same sample (Figure 3A) with a standard solution contain a mixture of 5 μM for each amino acid material (Figure 3B). The result clearly shows the concurrent peaks retention time of each individual amino acid with the corresponded peaks obtained from spiking the standard solution (see also Figure 1).

The detected amino acid levels in the tea grade known in the market as high grade tea types of black (Sam.1), oolong (Sam.6), green (Sam.8) and white (Sam.10) originated from Silane (SriLanka) are presented in Figure 4. Besides, a recovery test was performed for each of the analyte identified in the same four different tea types. This was done by spiking every tea infusion with 0.5, 1.0 and 5.0 μM of amino acid standards and repeating each spiking experiment three times. The results of the recovery study are presented in Table 8 and show that the average recoveries are ranged from 92.5 to 106.8%.

Table 8: Average recovery and RSD% of amino acids from tea samples, at various concentrations (n=3).

Amino acid	Spiked Concentration μM	Average recovery* ± RSD (%) n=3			
		Black tea Sam. 1	Green tea Sam. 6	Oolong tea Sam. 8	White tea Sam.10
Ala	0.5	99.4 ± 2.21	99.6 ± 2.44	98.8 ± 2.36	100.6 ± 1.97
	1	99.7 ± 1.98	98.5 ± 1.86	99.4 ± 2.47	99.6 ± 1.43
	5	100.0 ± 1.14	99.3 ± 1.11	99.0 ± 1.25	101.2 ± 0.97
Arg	0.5	105.2 ± 2.91	104.9 ± 2.46	105.4 ± 2.84	104.4 ± 2.38
	1	104.3 ± 2.28	105.2 ± 1.58	105.9 ± 2.03	105.7 ± 1.24
	5	106.8 ± 2.05	105.7 ± 1.28	106.1 ± 1.43	106.3 ± 1.67
Tyr	0.5	93.2 ± 2.47	92.5 ± 2.32	93.6 ± 1.98	93.1 ± 2.17
	1	93.7 ± 1.31	93.1 ± 2.43	94.8 ± 2.13	94.4 ± 1.61
	5	94.5 ± 1.15	94.7 ± 1.86	95.2 ± 1.70	93.1 ± 1.84

The profile of amino acids (Alanine, Arginine and Tyrosine) compositions for each tea type sample were determined and presented in Table 9.
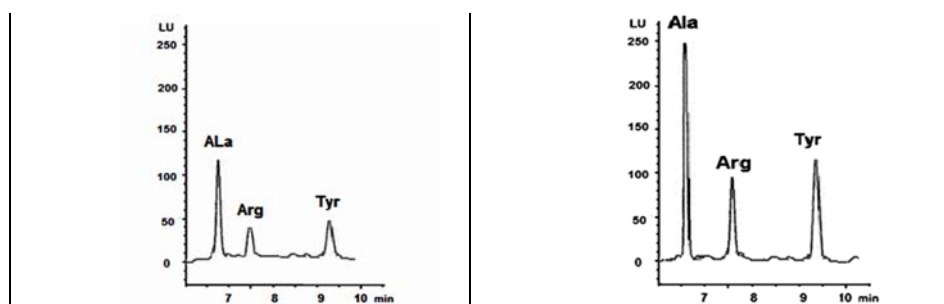
(A)	(B)

Figure 3: Chromatogram after pre-column derivations with OPA:3-MPA, applying the actuel HPLC conditions and FL detection for: (A) black tea infusion (Sam 1)
B) the same black tea infusion after spiking with amino acid mixture (5 µM of each).

Table 9: Amino acids content of black*, oolong**, green , and white†† teas; (n=5).

Samples	mean (± SD) (µg /g)		
	Ala	Arg	Tyr
Sam. 1	280 (6.06)	610 (16.15)	320 (5.67)
Sam. 2	330 (6.94)	670 (17.65)	310 (5.55)
Sam. 3	300 (6.30)	590 (15.79)	370 (6.53)
Sam. 4	190 (4.45)	550 (14.88)	310 (5.58)
Sam. 5	120 (2.85)	390 (10.68)	260 (4.72)
Sam. 6	330 (6.94)	490 (13.35)	240 (4.34)
Sam. 7	410 (8.26)	400 (10.81)	290 (5.19)
Sam. 8	200 (5.58)	530 (14.30)	390 (6.89)
Sam. 9	280 (6.06)	510 (13.83)	320 (5.68)
Sam. 10	1050 (16.74)	360 (9.99)	450 (7.86)
Sam. 11	1180 (18.53)	400 (10.90)	400 (7.01)

*Black tea: Sam. 1 (bags, Silane; Sri Lanka), Sam. 2 (bags, Indian), Sam. 3 (grade A; China), Sam. 4 (grade B; China), and Sam. 5 (grade C; China).
** Green tea: Sam. 6 (Silane; Sri Lanka), and Sam. 7 (bags; China),
†Oolong tea: Sam. 8 (bags, Silane; Sri Lanka), and Sam. 9 (bags, China).
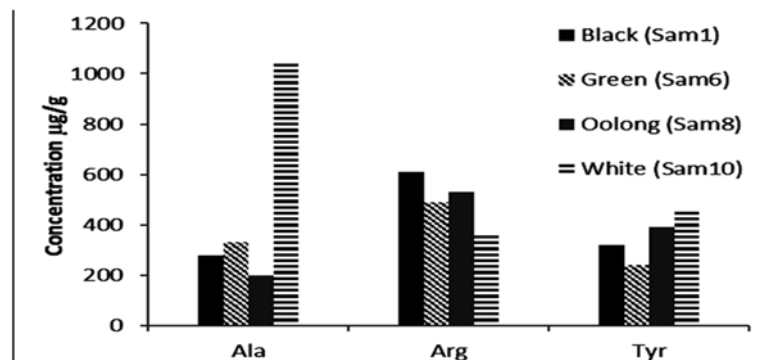††White tea: Sam. 10 (bags, Silane; Sri Lanka), and Sam. 11 (bags, China).

Figure 4: Amino acids profile for high grade tea types
originated from Silane (Sri Lanka).

The results show that Alanine clearly has the highest concentration in white tea, compared with black, green and oolong tea .

Black tea of different origin show relatively similar amino acid levels, where the grade B and C which consider in the market contain low tea quality, and show considerable lower amino acid levels as compared with the same origin of high Chinese quality (Grad A) or with Silane and Indian origin.

Conclusion.

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The described analytical method was involved the utilization of HPLC-Fluorescence with pre-column isoindole's derivations. The HPLC results clearly show the advantages of isoindole's derivations as in comparison with blank solution or amino acids free of derivation reagents. The developed method is validated and proved to be rapid, i.e. analysis time of about 10 minutes HPLC elution time is required for simultaneous separation of Alanine, Arginine, and Tyrosine without any interference, this is beside to the high sensitivity and excellent LOD response of nM levels.

Advantageous, the application of this method is to quick in the filed of classification of tea quality and to standardize the grades.

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