A GC-FID method for analysis of Lysinoalanine

Antonia Montilla, José Ángel Gómez-Ruiz, Agustín Olano, María Dolores del Castillo

Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Científicas, Madrid, Spain.

Running title: GC analysis of LAL

Correspondence: Dr. Dolores del Castillo, Instituto de Fermentaciones Industriales, Consejo Superior de Investigaciones Científicas, c/ Juan de la Cierva 3, 28006 Madrid, Spain

E-mail: delcastillo@ifi.csic.es

Fax: +34915644853

Abbreviations: **LAL**, lysinoalanine; **BSA**, bovine serum albumin; **DPA,** diaminopimelic acid; **MTBSTFA**, *N*-methyl-*N*-*(tert*-butyldimethylsilyl)-trifluoracetamide; **TEA,** triethylamine; **tBDMSi**, *N*-*(O)*-*tert*-butyldimethylsilyl; **FID**, flame ionization detector

Keywords: Lysinoalanine, Gas-chromatography, *N*(O)-*tert*-butyldimethylsilyl derivatives.

ABSTRACT

Lysinoalanine (LAL) is an unwanted by-product, which is formed during processing of protein and protein-containing foods and feeds. A GC method for quantitative analysis of LAL under conventional chromatographic conditions has been developed. The method was applied to the analysis of pure standard substances, boiled eggs, commercial caseinates, fresh cheeses, fresh cheeses made with milk supplemented with caseinate and fresh cheeses adulterated with caseinate after cheese making process. Results demonstrated the reliability of the GC capillary chromatography for the analysis of LAL in protein containing foods. Limits of detection and quantification of 50 ppm and 152 ppm of LAL in protein, respectively, were achieved. Range of linearity, precision and accuracy of the method, measured using diaminopimelic acid as internal standard, were satisfactory for quantification purpose. The method might be also suitable for the quantitative analysis of others amino acids such as lysine and arginine. Results also indicated the utility of this methodology for detecting protein quality of egg products and caseinates as well as fresh cheese frauds.

1 Introduction

Lysinoalanine [LAL, N^{ε}-(*R*, *S*-2-amino-2-carboxyethyl)-*S*-lysine] has been used as a marker of thermal damage in foods. The mechanism of formation of this cross-linking amino acid takes place in two main steps. The first step consisting in the formation of dehydroalanine residue from the *β*-elimination of cystine, serine or its derivatives (*O*-phosphorylserine and *O*-glycosylserine) which is an hydroxide ion-catalyzed reaction, followed by a second reaction involving the double bond of dehydroalanine with a nucleophilic side chain of another amino acid, such as the ε-amino group of lysine [1-2]. Formation of LAL along the protein chain may affect the nutritional and biological properties of the treated proteins. Decreasing of essential amino acids and reducing of protein digestibility, protein quality, mineral bioavailability and utilization, within other nutritional and toxicological effects, are some of the negative consequences derived from LAL generation [1-2]. High pH, temperatures, and long exposure time make these transformations favorable. Thermal treatments at basic pH are quite common in the food industry. Therefore, LAL may be found in widely consumed foods such as, baby food, cereal products, chicken meat, egg products, gelatine, infant formulas, meat products, caseinate, soy protein isolate, liquid milk, powdered milk, cheese among others [1, 3-16]. Significant concern arises with the detection of LAL in special foods, such as enteral nutrition formulas, where the quality of the protein used in their formulation is particularly relevant because these foods may be the sole nutrient source for patients during long periods of time [17]. In addition, LAL has been considered a better quality marker than Maillard reaction products for assessing the nutritional quality of proteins which are widely employed as ingredient in the food industry, for instance caseins [9]. Therefore, strategies to minimize LAL formation during food processing and easy methods for its routine determination need to be developed.

Various analytical procedures have been evaluated for determining LAL in protein and protein-containing foods and feeds. Because LAL survives the acidic conditions of proteins hydrolysis

commonly used for the analysis of amino acids [1], its determination can be performed by Ion-Exchange Chromatography (amino acid analyzer) with colorimetric and fluorometry detection after an acid protein digestion [18-19], GC analysis of n-butyl esters of *N(O)*-trifluoroacetyl derivatives [6, 20-21], GC-MS analysis of diasteromeric *N*(*O,S*)-perfluoropropionyl isopropyl esters [22] or TLC and HPLC analysis [14, 23-26]. Most of the data recently published related to LAL analysis in foods have been acquired by the HPLC method proposed by Pellegrino *et al*. [9], based on derivatization with FMOC-Cl, solid phase extraction, reverse phase chromatography and fluorescence detection. The method is very sensitive (0.5-1 μg/g protein equivalent to ppm in protein); however, the sample preparation is complex and time consuming. The aim of this investigation was to find out a feasible procedure to achieve an easy and specific determination of LAL in the range of concentration commonly detected in protein containing foods. A GC-FID method has been developed to achieve this purpose based on previous studies related to LAL analysis by GC [6, 20] and glycated and ascorbylated proteins by GC-MS [27].

2 Materials and methods

2.1 Chemicals

All chemicals used were of analytical grade. LAL was obtained from Bachem AG (Bubendorf, Switzerland). Bovine serum albumin (BSA), DL-2,6-diaminoheptanedioic acid also called diaminopimelic acid (DPA) and *N*-methyl-*N*-*(tert*-butyldimethylsilyl)-trifluoracetamide (MTBSTFA) were from Sigma (St. Louis, USA). Hydrochloride acid was supplied by Panreac (Barcelona, Spain). Triethylamine (TEA) and *N*, *N*-dimethylformamide (DMF) were from Merck (Darmstadt, Germany). High purity water was produced in-house using a Milli-Q Synthesis A10 System (Millipore, Bellerica, Mass., USA) and was used throughout.

2.2 Food samples

2.2.1. Boiled eggs

Grade A chicken eggs, medium size (53-63 g), laid by 7-8 days were obtained from a local farm and heated for 10, 20, 30, 45 and 60 min. Starting temperature was 20-25ºC, after 10 min of heating 79-82ºC were achieved and water boiling point arrived by 14 min. Shelling and separation of egg albumen from yolk were done carefully and manually. Samples were stored at –20ºC until analysis. Treatments were carried out in triplicate.

2.2.2. Caseinates

Sodium (n=2) and calcium caseinates (n=3) were supplied by three European dairy companies.

2.2.3. Fresh cheeses

Raw cow milk from a local farmer was employed for cheese-making. Previous to the manufacture of the cheeses, the raw milk was pasteurized at 65 ºC for 30 min.

Three different batches were prepared in triplicate. A first batch was prepared without calcium caseinate (A, control). The second batch (B) was based on fresh cheeses from pasteurized milk supplemented with calcium caseinate to reach a final concentration of 15 g/L in milk. Briefly, after warming up the milk to 33ºC, a lactic starter was added and the milk stirred for 35 min to help the acid development. Subsequently, 0.025 g/L calcium chloride were added and the milk stirred for another 5 min. Finally 0.05 g/L of rennet were added. After 60 min of coagulation, the curd was cut and the whey drained from the cheese. This step was accompanied with heating the curd at 38ºC for 20 min and gentle stirring followed by a first centrifugation step at 1700 *g*, 23ºC for 60 min. The purpose of these stages of the process was to increase the rate at which the curd contracts and squeezes out the whey making a hard curd. Once the curds had sufficiently hardened, salting for 30 min was performed. A second centrifugation step at described above was carried out. Then, cheeses were cut, stored in the fridge overnight, vacuum packed and frozen at –20ºC until analysis.

A third batch of cheese samples consisting in fresh cheeses made as described above (A) to which 2.5, 5, 10 and 15% of commercial calcium caseinate containing 605.0 ppm of LAL in protein was added (C1, C2, C3, C4) was also studied in the present paper. Adulterated cheeses were stored at –20ºC until analysis.

2.3 GC analysis

2.3.1 Acid hydrolysis

Samples containing 40-50 mg of protein and DPA (160 µg) as an internal standard (I.S.) were thermally digested by 8 ml of 6 M HCl in Pyrex glass bottles at 110ºC for 23 h. Prior incubation samples were degassed using a stream of helium for 2 min. The hydrolyzed samples were cooled at room temperature and filtered through a Whatman 40 paper filter.

2.3.2 Derivatization

Aliquots of filtrated hydrolyzates (0.5 ml) were evaporated to dryness at 38-40ºC with a vacuum rotary evaporator. After evaporation, derivatization of the dried samples was carried out according to the methods of Hasenkopf *et al*. [27] and Woo and Chang [28]. DMF (165 µl) and TEA (15 µl) were added to the dry sample and stirred for 1 min. Afterwards, 100 µl of MTBSTFA, silylating reagent, were added and the reaction mixture was heated at 70ºC for 60 min. Derivatization reaction was stopped by cooling to room temperature and the samples containing *N-(O)-tert*-butyldimethylsilyl (tBDMSi) derivatives of the amino acids were injected onto the GC column.

2.3.3 GC-FID analysis

The chromatography was performed by using a Hewlett-Packard HP6890 (Waldbronn, Germany) gas chromatograph equipped with a flame ionization detector (FID). A CP-SIL 5CB commercial fused silica capillary column (100 % bonded dimethylsiloxane, 25 m x 0.25 mm id, 0.25 µm film thickness) (Chrompack, Middelburg, The Netherlands) was used. The carrier gas (nitrogen) flow-rate was 1.2 ml/min. The make up gas was also nitrogen at flow-rate of 20 ml/min. Injector and detector temperatures were 280 and 300ºC, respectively. For analysis, 3 µl of tBDMSi amino acid

derivatives were injected in split-less mode. The oven temperature was programmed from 100ºC, held 1 min, ramp to 250°C at 30°C/min, held for 42 min, ramp to 300°C at 50°C/min and held for 5 min. Data was acquired by means of HP ChemStations (Agilent Technologies Inc., Wilmington, USA).

The range of linearity of FID response was checked by employing a calibration curve of LAL. The calibration curve was constructed by adding different amounts of LAL to a standard mixture containing known quantities of BSA and DPA to obtain final concentrations of LAL from 152 to 3800 ppm in protein. The mixtures were hydrolyzed, derivatized and the derivatives injected on to the column.

tBDMSi LAL derivative was quantified by internal standard method. All analyses were carried out in duplicate and the data were the mean values expressed as mg LAL / Kg protein (ppm in protein)**.** Total nitrogen was determined by means of Kjeldahl method [29, 30] and the protein values were calculated using 6.25 and 6.38 as conversion factors for eggs and dairy products, respectively.

LAL peak identification was achieved by migration time, standard addition and mass spectrometry analysis.

2.3.4. GC-MS analysis

A HP-6890 chromatograph coupled to a MD 5973 quadrupole mass detector (Hewlett-Packard, Palo Alto, CA, USA) was employed. The separation of the tBDMSi amino acid derivatives was performed on a 30 m x 0.25 mm id x 0.25μm film thickness, HP-5MS (bonded 5% phenyl, 95% dimethylpolysiloxane) fused silica capillary column from Agilent Technologies Inc. (Wilmington, USA). Helium was used as carrier gas. The oven temperature was programmed from 80ºC, held 1 min, ramp to 200ºC at 50ºC/min, ramp to 250ºC at 10ºC/min, held for 42 min, ramp to 300ºC at 50ºC/min and held for 5 min. Samples were injected in split-less mode. Mass spectrometer was operated in EI mode at 70 eV. Mass spectra were acquired using a G1701CA ChemStation Software (Hewlett-Packard, Palo Alto, CA, USA).

2.4.4. Statistical analysis

Statistical analysis (Microsoft Excel 2000) of data was performed by one factor analysis of variance with a level of significance of 95%.

3 Results and discussion

3.1 GC setup

Figure 1 shows the chromatogram of tBDMSi amino acid derivatives of hydrolyzed BSA spiked with pure LAL to a final concentration of 3800 ppm in protein, which is split up into two zones to provide a better look of the GC-FID profile. tBDMSi-DPA eluted at 23.03 min while tBDMSi-LAL showed a retention time of 48.61 min corresponding to a relative retention time of 2.11. tBDMSi-LAL peak was not detected in untreated/non spiked BSA hydrolyzates. Results indicated that DPA is a suitable internal standard for the determination of LAL by GC-FID agreeing with data previously described by Hasegawa *et al*. [6]. Purification of the sample was not necessary and stable baseline during the analysis was obtained.

As can be observed in Figures 1A and 1B, both tBDMSi-DPA and tBDMSi-LAL were very well separated from the derivatives of the common twenty amino acids of proteins such as tBDMSi-Lysine (relative retention time of 0.36) and tBDMSi-Arginine (relative retention time of 0.43). Most of the 20 amino acids found in proteins eluted within 20 minutes of analysis with the exception of cystine, which showed a retention time of 47.2 min equivalent to a relative retention time of 2.05. Since chromatographic interferences have not been observed simultaneous analysis of arginine, lysine and LAL might be carried out under the proposed conditions.

tBDMSi-LAL was also analyzed by GC-MS. For this compound, one peak appeared in the chromatogram corresponding to a derivatization product with four silyl groups. Chemical structure and mass spectrum of tBDMSi-LAL are shown in Figure 2. The most common MS fragments found in EI mass spectrum of the tBDMSi derivative of LAL agreed with those previously described by Hasenkopf *et al*. [27] for others tBDMSi derivatives of amino acids. The [M^+] ion was not identified. Instead [M-

57]$^+$ ion, which corresponds to the LAL with four silyl groups and the loss of $C(CH_3)_3$ was detected. Ion with m/z 387 [M-159-131-15]$^+$ due to a simultaneous loss of COOtBDMS, OtBDMS and CH_3, can also be observed in Figure 2 as the most prominent fragment.

Detection and quantification limits were determined as three and ten folds the S/N (signal/noise) ratio near the retention time of LAL, respectively. Detection and quantification limits of 50 ppm in protein and 152 ppm in protein, respectively were obtained. Sensitivity for the determination was lower than that reported by HPLC (~1 ppm in protein) [9] and similar to those reported using GC-NPD (nitrogen-phosphorus detection) [20] and ion-exchange chromatography (≥ 100 ppm in protein) [31]. The results suggested that the method provides the necessary selectivity avoiding a previous clean up step of the sample. In widely consumed foods such pasteurized, UHT and sterilized milks LAL values ranged from 17 to 69, 49 to 186 and 224 and 653 ppm in protein have been measured [14]. Upper limits of 200 ± 100 and 1000 ppm of LAL in protein for dried and liquid products, respectively, have been legally established in Germany for special foods like infant products [14, 32] and also recommend by the European regulatory organization [33]. Moreover, a low LAL content in foodstuffs (<500 ppm in protein) has been recommended in order to obtain positive benefit from the nutritional point of view [34], amounts which are measurable by the proposed method. Most of the harmful effects associated to LAL have been caused by daily ingestion of doses higher than 500 ppm in protein [1].

Figure 3 shows a LAL standard curve ranged from 152 to 3800 ppm in protein. A linear relationship (y=3.6624x + 0.0155) between the response measured as peak area and concentration of LAL over this range was observed. The regression coefficient of the curve was 0.9859. One factor analysis of variance of the response factor of LAL demonstrated significant homogeneity of the variance between the samples over the range of concentration studied since a *F*-value of 0.7327 with *P*=0.8235 ($P > 0.05$) was obtained. Data agreed with those reported by Hasegawa *et al*. [6]. The precision of the method was estimated by analysis of BSA sample spiked with 380 ppm of LAL in protein within the same day (repeatability, n=3) and in different days (intermediate precision, n=4),

obtaining relative standard deviations of 5.23 and 7.75, respectively. Recovery of the method was also evaluated by adding known amounts of LAL to pure BSA, raw albumen and fresh cheese (A). Average values of 94.0 %, 90.2% and 91.1% of LAL were recovered from hydrolyzates of BSA, raw albumen and fresh cheeses, respectively.

Although the GC analysis of tBDMSi-LAL has been previously proposed, this is the first time that capillary columns commercially available are used. The use of commercial GC columns could facilitate the development of standardized procedures. The method here reported offers significant improvements with respect to previous methods based on GC analysis of tBDMSi-LAL in terms of simplicity, time of analysis and resolution. In addition, it allows simultaneous quantitative determination of LAL and the common twenty amino acids found in proteins, among them lysine, which is an essential amino acid considered as a chemical indicator for control of protein nutritional quality in processed foods.

3.2. Analysis of LAL in food samples

To study the applicability of the validated method the presence or formation of LAL in different food matrices (boiled eggs, caseinates and fresh cheeses) was evaluated.

3.2.1. Crosslinking during boiling of eggs

Table 1 shows the concentration of LAL found in fresh and boiled albumen and yolk. No LAL was observed in raw samples. LAL content increased progressively as a function of the heating treatment time. Rate of LAL formation in boiled white was faster than that detected in the boiled yolk egg. Similar levels of LAL in both, albumen and yolk, have been found by Hasegawa *et al*. [6].

3.2.2 LAL in commercial caseinates and experimental fresh cheeses

LAL values up to 605.0 ppm in protein were detected in commercial caseinates. Data agreed with those previously reported by others authors [1, 10]. GC profile corresponding to calcium caseinate, which showed the highest LAL content within the samples under study, is presented in Figure 4A.

Table 2 shows the LAL content of both fresh cheeses and fresh cheeses made from milk supplemented with calcium caseinate. Chromatograms of both cheeses are shown in Figure 4B and 4C. No LAL was detected in fresh cheeses (Figure 4B and Table 2). No LAL or quantities of this compound lower than 69 ppm have been reported in pasteurized milk [1, 14]. LAL is not a suitable marker for milks but it is for caseins and caseinates [10]. In cheeses based on milk supplemented with 15g/L of calcium caseinate (B), values from 140.2 to 207.6 ppm of LAL in protein were detected (Table 2). Differences in LAL contents might be due to a differential rate of caseinate absorption during cheese making which is in agreement with protein data. Higher values of LAL corresponded to higher protein contents (Table 2).

Adulteration of fresh cheeses with amounts of caseinates ranged from 2.5% to 15% (C) was detected by employing the GC methodology here described. A good relationship between the expected and the experimental LAL data was obtained. Calculated values for LAL in fresh cheeses adulterated with 10 % (167.1 ppm in protein) and 15 % (208.8 ppm in protein) of caseinate agreed with those expected (179.8 and 234.4 ppm in protein, respectively) suggesting the feasibility of the method to detect cheese adulterations with caseinates like Mozzarella substitutes (1,9-10, 16).

4 Concluding remarks

The proposed procedure allows the quantitative analysis of LAL by gas capillary chromatography employing conventional conditions without complex and time-consuming preparation. In addition, lysine, arginine and others amino acids may be analyzed employing the proposed methodology. Results indicated the feasibility of this analytical tool for detecting protein quality of egg products, caseinates and fresh cheese frauds.

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Figure legends

Figure 1. GC chromatogram of tBDMSi amino acid derivatives of hydrolyzed BSA spiked with pure LAL. GC profile is split up into two zones as follows: Panel A shows those peaks corresponding to the tBDMSi derivatives DPA, DL-2,6-diaminoheptanedioic acid (internal standard) and LAL, lysinoalanine while in Panel B can be observed the derivatives of arginine, lysine and DPA.

Figure 2. Chemical structure and mass spectrum of tBDMSi derivative of pure LAL obtained by electron impact ionization GC-MS.

Figure 3. Relationship between response (Areas) ratio and weight ratio of tBDMSi-LAL and tBDMSi-DPA obtained by capillary GC-FID analysis.

Figure 4. GC-FID profiles of tBDMSi amino acid derivatives of hydrolyzed commercial calcium caseinate (A), fresh cheese (B) and fresh cheese made from milk containing 15 g/L of calcium caseinate (C).

Table 1. LAL contents formed by boiling eggs. Results are expressed as mean ± S.D. (n=3).

Heating conditions	LAL content (ppm in protein)	
	Egg albumen	Egg yolk
Raw	N.D	N.D
79-82ºC,10 min	traces	N.D
Heating 20 min (6 min boiling)	153.3 ± 26.6	N.D
Heating 30 min (16 min boiling)	211.0 ± 25.4	traces
Heating 45 min (31 min boiling)	622.4± 58.7	traces
Heating 60 min (46 min boiling)	677.0 ± 63.4	154.4 ± 28.5

N.D.= no detected

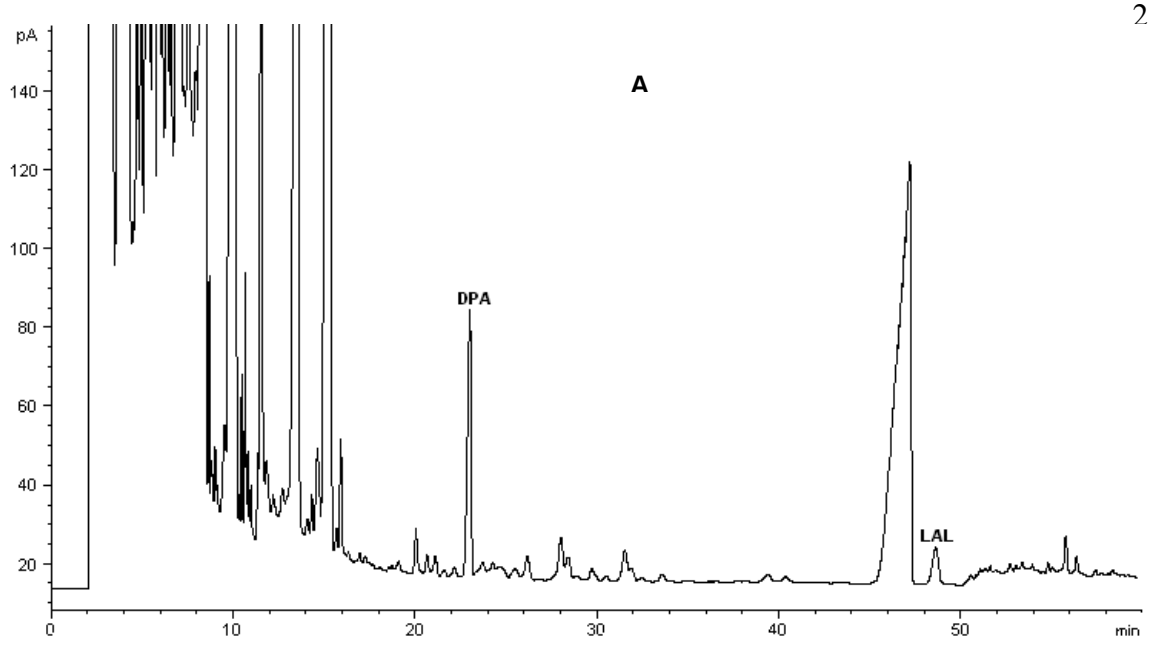
1 Table 2. Protein and LAL contents of experimental fresh cheeses. A, control fresh cheeses, B, fresh
2 cheeses made from milk supplemented with 15g/L of caseinate. Results are expressed as mean ± S.D
3 (n=3).

Sample	LAL (ppm in protein)	Protein (g/100 g of product)
A1	0	20.9
A2	0	21.6
A3	0	21.1
B1	158.9±26.5	22.3
B2	207.6±18.6	23.1
B3	140.2±9.5	21.3

4

1 Figure 1.

2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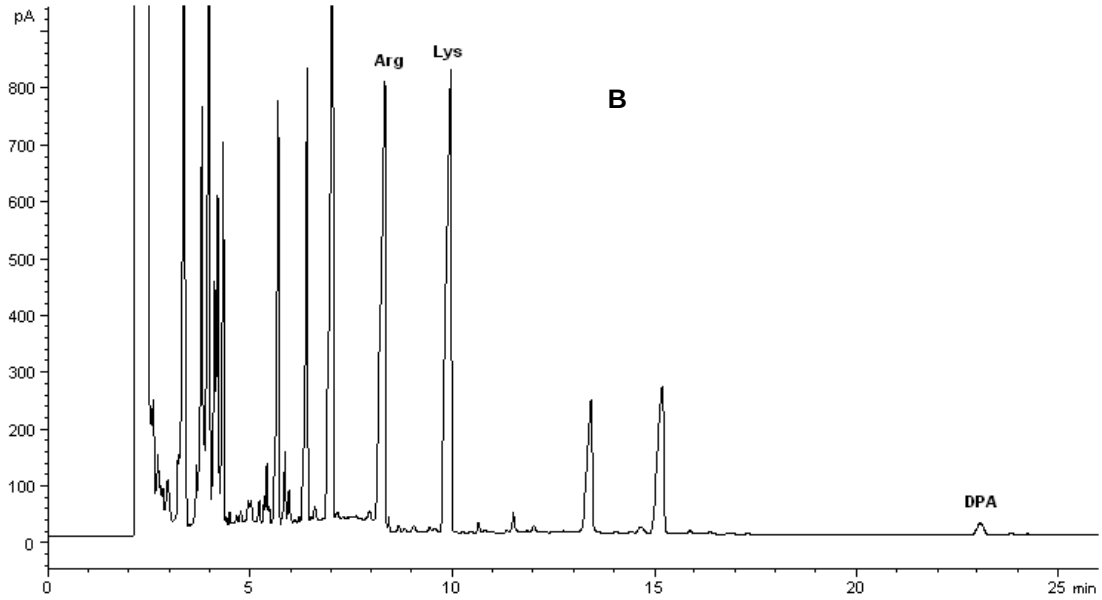
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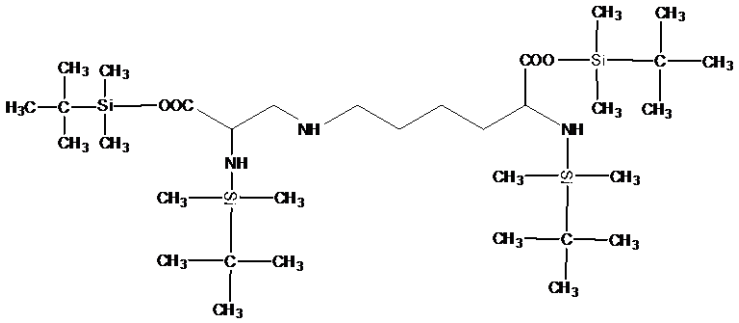
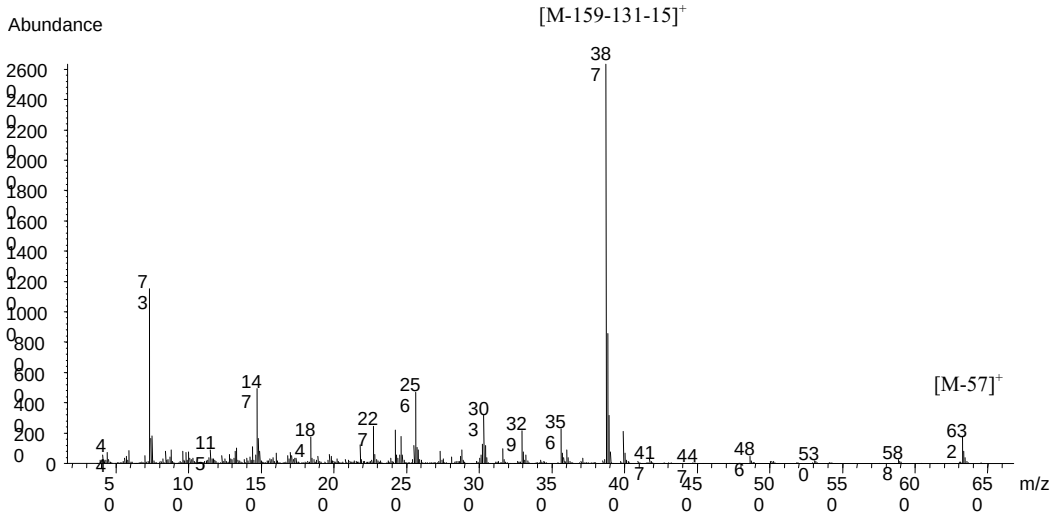
1 Figure 2

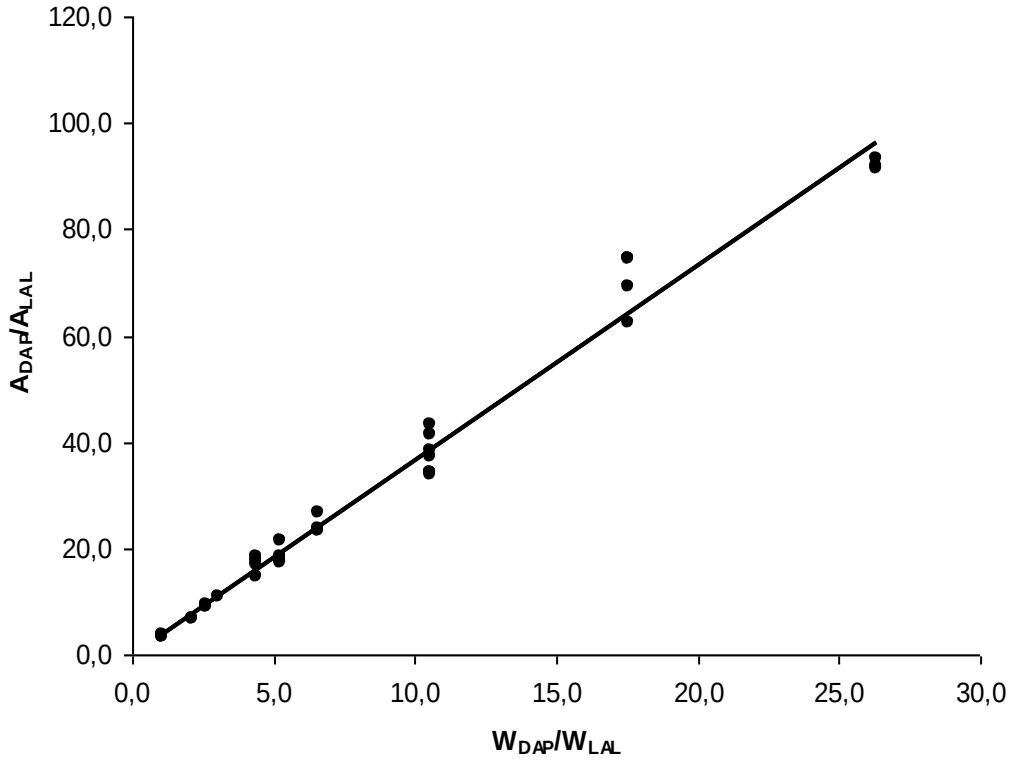
2





1 Figure 3.

2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100,0
80,0
60,0
40,0
20,0
0,0
A_{DAP}/A_{LAL}
0,0
5,0
10,0
15,0
20,0
25,0
30,0
W_{DAP}/W_{LAL}

1 Figure 4

2