ANALYSIS OF ETHYL-N$^{\alpha}$-LAUROYL-L-ARGINATE HCl IN SOLID AND SEMI-SOLID FOOD MATRICES

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1. BASIS

This method describes the necessary steps to determine the residual content of the active ingredient in LAE (ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl) in solid and semi-solid food matrices. The type of the food matrix from which ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is extracted determines which method is going to be used.

Once ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is extracted, it is analysed by Reserve Phase High Performance Liquid Chromatography (HPLC-RP) and quantified using an external standard curve. This method of analysis describes two conditions of chromatographic analysis, one using isocratic conditions and the other one using gradient conditions. The use of one of them depends on the type of food matrix that is going to be analysed. Thus, it is convenient to have control samples (without ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl content) in order to check the absence of chromatographic interferences in the analytical conditions studied. The examples studied have demonstrated that gradient conditions were more efficient than isocratic conditions and for this reason gradient conditions are recommended.

Solid and semi-solid food matrices from where this method was successfully applied are:

- Cooked meat products: cooked ham, Frankfurt and Bratwurst sausage, nuggets
- Raw meat products: hamburguers, ground pork, minced meat, steak, fresh sausages, chicken breasts
- Meat toppings for pizza
- Cold crabmeat
- Tripe in sauce
- Skin of the chicken thigh (for this matrix an special method has been developed, ID-11-2392)
- Rice
- Bechamel sauce (butter, flour and milk)
- Chickpeas
- Fried tomato sauce

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LAS, which comes from the hydrolysis of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, is also detected in this method of analysis. Although quantification of LAS is possible, it is not the goal of this analysis.

2. INTERFERENCES

In the assayed studies, no interferences have been found in the analysis except the matrix itself.
If the level of sulphites is high, chromatographic interferences could be observed (the HPLC-RP column could be damaged). In these cases, it is advisable to add to the sample a little amount of hydrogen peroxide[*].

3. REAGENTS

- Chloroform, analysis grade
- Methanol, analysis grade
- Acetonitrile (ACN), HPLC grade
- Water, Mili Q® Academic Ultrapure grade
- Trifluoroacetic acid (TFA), synthesis grade
- Ethyl-N$^{\alpha}$-L-arginate HCl standard (Batch Q-98.250, purity ≥95%)
- LAS, ethyl-N$^{\alpha}$-Lauroyl-L-arginine (Batch Q-98.251, purity ≥98%)

MATERIALS

- Extraction thimbles, 30 mm x 80 mm
- Analytical balance, MC1, precision ≥± 0.1 mg
- Centromix Centrifuge, ≥ 4000 rpm
- High Performance Liquid Chromatography (Waters)
- Volumetric flasks and measuring pipettes, A class
- Nylon filters, 0.45 µm
- Magnetic stirring rods
- Homogeneizer
- Stomaker
- Bag filters for Stomaker

[*] In these analysis, a sample with 500 ppm of LAE a.i. and 5000 ppm of metabisulphite has analysed. The analyte has been extracted with ACN + 0.1% TFA and 84 µL of hydrogen peroxide (purity 30%) has been added to 2 mL of the extract. The reaction is fast and virulent.

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4. CHROMATOGRAPHIC CONDITIONS

The most suitable detector for this type of analysis is the photodiode detector at the range of wavelength between 190-300 nm.

4.1. Isocratic chromatographic conditions:

Column: Symmetry® C_{18} 5 µm 150 x 3.9 mm (Waters)
Solvent: ACN/H_2O (50/50 v/v) + 0.1% TFA[1]
Flow rate: 1 mL/min
Wavelength: 215 nm (range: 190-300 nm)
Injection volume: 10 µL
Retention time (R_T): ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl; 4.3 minutes (± 0.2)[2];
Retention time (R_T): LAS: 2.3 minutes (± 0.2)[2]
Total time: 10 minutes

4.2. Gradient chromatographic conditions:

Column: Symmetry® C_{18} 5 µm 150 x 3.9 mm (Waters)
Solvent A: H_2O + 0.045% TFA[3]
Solvent B: ACN + 0.036% TFA[4]
Wavelength: 215 nm
Injection volume: 20 µL
Retention time ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl; 19 minutes (± 0.3)[5]
Retention time LAS: 16.2 minutes (± 0.3)[5]
Total time: 40 minutes (32 minutes of gradient + 8 minutes of equilibration)

Time (minutes)	Flow (mL/min)	% A	%B
0	1.0	70	30
5	1.0	70	30
25	1.0	30	70
27	1.3	0	100
30	1.3	0	100
32	1.0	70	30

[1] 500 mL of H_2O + 500 mL of ACN + 1mL TFA.
[2] The retention time for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS were obtained at LAMIRSA laboratory under the reported chromatographic conditions. The indicated interval is due to R_T variations observed when the concentration of analyte was altered.
[3] 1L H_2O + 450 µL TFA.
[4] 1L ACN + 360 µL TFA.
[5] The retention time for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS were obtained at LAMIRSA laboratory under the reported chromatographic conditions. The indicated interval is due to R_T variations observed when the concentration of analyte was altered.

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5. PROCESS

5.1. Preparation of Standard Solutions

Standard ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and LAS solutions are prepared by adding powdered substrate to ACN/H_2O (50/50 v/v + 0.1% TFA). The range of substrate concentrations used to create a standard curve must bracket the substrate concentration expected to be present in the analysed food sample. At least, four different substrate concentrations ("standards") must be prepared and analysed to create the standard curve. The volume injected will be 10 μL.

5.2. Sample Preparation[6]

Due to unpredictable chromatographic interferences from new food matrices treated with ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl, the availability of different methods of sample preparation is required. It is difficult to determine *a priori* which method of sample preparation will completely extract residual ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl from a new food matrix to be analysed. In order to make this determination, a known concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is added to a sample of the food matrix of interest. Methods A and B can then be both employed for the extraction of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl and the method which maximally recovers the known quantity of the active ingredient is determined.

The suggested mass and volume values presented in this analytical method are appropriate for extracted ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl concentrations in the range of 10 to 1,000 mg/L.

5.2.1-Method A: direct analysis from a solid and semi-solid food matrix

1/ Historically, the method developed was: a representative sample of food for the analysis of residual ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl is homogenized using a food grinder. Approximately 20 mL of ACN + 0.1% TFA is added to approximately 12 g of the ground sample. In a closed container[7], at room temperature and in the dark, the sample and

[6] The values of mass and volume reported in this document are only indicatives and suitable for the analysis of a food matrix with an approximate concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl equal to 77 mg/kg. Mass, volumes and conditions used for analysis may need to be adjusted for each new case.

[7] The extraction process must be performed in a closed container in order to avoid the loss of solvent by evaporation.

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solvent are stirred for approximately 17 hours using a magnetic stirring bar. Using a plastic syringe, an aliquot of approximately 5 mL is removed from the liquid fraction and then filtered through a 0.45 µm nylon filter. This filtered sample is then analysed using the appropriate chromatographic method.

For food matrices with low water content is more suitable to employ ACN/H_2O (50/50 v/v) + 0.1% TFA for the ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl extraction.

2/ Afterwards, a new method for the extraction of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl has been developed for solid and semi-solid food matrices:

Weigh a known sample quantity, approximately 20 grams. In a Stomaker, ground the sample three times, 3 minutes for each one, with the appropriate solvent to complete a final volume of 50 or 100 mL.
When the extraction has been finished, with a disposable syringe, extract a fraction of the sample and filter it with a filter of nylon 0.45 µm and inject 10µL.

Matrix	Solvent	Sample weight	Stomaker time (min)	Final Volume (mL)	Cromatographic conditions
Frankfurt sausage	ACN + 0.1% TFA	10-20 g	3+3+3	50-100	Gradient
Bratwurst sausage	ACN + 0.1% TFA	10-20 g	3+3+3	50-100	Gradient
Cooked ham	ACN + 0.1% TFA	10-20 g	3+3+3	50-100	Gradient
Steak	ACN + 0.1% TFA	10-20 g	3+3+3	50-100	Gradient
Marinated meat	ACN + 0.1% TFA	10-20g	3+3+3	50-100	Gradient
Fried tomato sauce	ACN + 0.1% TFA	10-15	3+3+3	50	Gradient

3/ Food matrices which are difficult to stir:

Mixing certain food matrices with the magnetic stir bar may be difficult. In these cases, it is better to perform the extraction using a sonicator. Four examples and sonication method parameters are shown in the table below:

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Food Matrix	Sample Mass (g)	Solvent	Solvent volume (mL)	Extraction Conditions	Chromatographic conditions
Tripe in sauce	3-5	ACN+H_2O 50/50(v/v)+0.1% TFA	10	60 minutes at 35ºC	Isocratic
Minced meat	10	ACN+0.1% TFA	10	60 minutes at room temperature	Gradient
Chickpeas	10-20	ACN+H_2O 50/50(v/v)+0.1% TFA	50	2-4 hours at room temperature	Gradient
Bechamel	2	ACN+H_2O 50/50(v/v)+0.1% TFA	3	1 hour at room temperature	Gradient

Once the extraction process is completed, approximately 5 mL of the liquid fraction is collected with a plastic syringe, filtered through a 0.45 µm nylon filter, and then analysed using the appropriate chromatographic method.

5.2.2-Method B: extraction from a solid food matrix by Soxhlet[9]

Remove all water from the sample by means of lyophilization using the following procedure.

Homogenize a representative piece of food to be analysed for ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl content. Into a tared plastic bag, weight the homogenized sample and spread it well to maximize its surface area. Freeze the sample at –15ºC (or colder) for at least 8 hours and 0.075 to 0.750 torr. The lyophilization process is complete when the weight of the sample is constant for two consecutive days.

Note: It is important to determine the weight of the sample before and after lyophilization.

Once the sample has been lyophilized, mix it very well and place approximately 12 g of the sample inside a 30 mm x 80 mm extraction thimble. The extraction process is performed in a Soxhlet apparatus using

[9] The values of mass and volume reported in this document are only indicatives and suitable for the analysis of a food matrix with an approximate concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl equal to 77 mg/Kg. Mass, volumes and conditions used for analysis may need to be adjusted for each new case.

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approximately 175 mL of a chloroform/methanol mixture, (80/20, v/v) for 8 hours. The extract obtained is then concentrated in a rotatory evaporator at approximately 14 torr. The extract is mixed with approximately 10 mL of ACN/H_2O (50/50 v/v) + 0.1% TFA. This suspension is then centrifuged at 4,000 rpm for 20 minutes in order to separate the hydrophobic phase from the hydrophilic phase. The hydrophobic phase is discarded and the hydrophilic phase, which is practically transparent, is filtered through a 0.45 µm nylon filter. The filtered sample is then analysed using the appropriate chromatographic method.

6. QUANTIFICATION

A standard curve is created with at least 4 standard concentrations bracketing the expected ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl concentration in the food matrix and plotting standard concentration *versus* peak area. Chromatographic conditions are optimised by means of the instructions from the chromatographic software. The standard curve must have a correlation coefficient (r^2) > 0.998 and the slope must not be different from that derived from a minimum of two standard curves prepared under the same conditions with a percent relative standard deviation (% RSD) < 2%. If these two conditions were not fulfilled, then the standard curve must be discarded and a new standard curve with freshly prepared standards must be created.

The concentration of residual ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in the analysed food is calculated depending on the method of analysis as shown below.

Direct extraction (method A)

$$C_{LAE} = \frac{C_{LAE\ obt}\ x\ V}{W}$$

C_{LAE}: Residual concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl found in the analysed food (mg/kg)

$C_{LAE\ obt.}$: Concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in the analysed phase (mg/L)

V: Total volume of solvent used for extration (L)

W: Mass of the treated sample (kg)

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Soxhlet extraction (method B)

$$C_{LAE} = \frac{C_{LAE\ obt}\ x\ V\ x\ W_2}{W_1\ x\ W_3}$$

C_{LAE}: Concentration of residual ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl found in the analysed food (mg/kg)
$C_{LAE\ obt}$: Concentration of ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl in the analysed phase (mg/L)
V: Total volume of solvent used for extration (L)
W_1: Mass of aliquot of the extracted lyophilized sample (kg)
W_2: Mass of the sample after lyophilization (kg)
W_3: Mass of the wet sample (kg)

Note: Any additional dilutions employed must be included in the final quantification calculation.

7. TECHNICAL NOTES

Analysis of food samples with a high fat content, such as pâté, must not be performed using the methods presented here because the chromatographic column can be irreversibly damaged by fatty acids. In this case, a pre-column should be employed in order to eliminate the impurities.

In those analysis that samples contain high levels of metabisulphites is possible that some interferences are produced at chromatographic level. In this case, the sample that is going to be analysed by HPLC-RP should be treated with a small content of oxygenated water[10].

[10] In these analysis, a sample that contained 500 ppm LAEai in front of 50000 ppm of metabisulphite in aqueous medium was treated. The analyte was extracted with ACN+0.1%TFA and with a volume of 2.0 mL of analyte extracted it has been added 84 μL of H_2O_2 at 30%. The reaction is virulent and fast.

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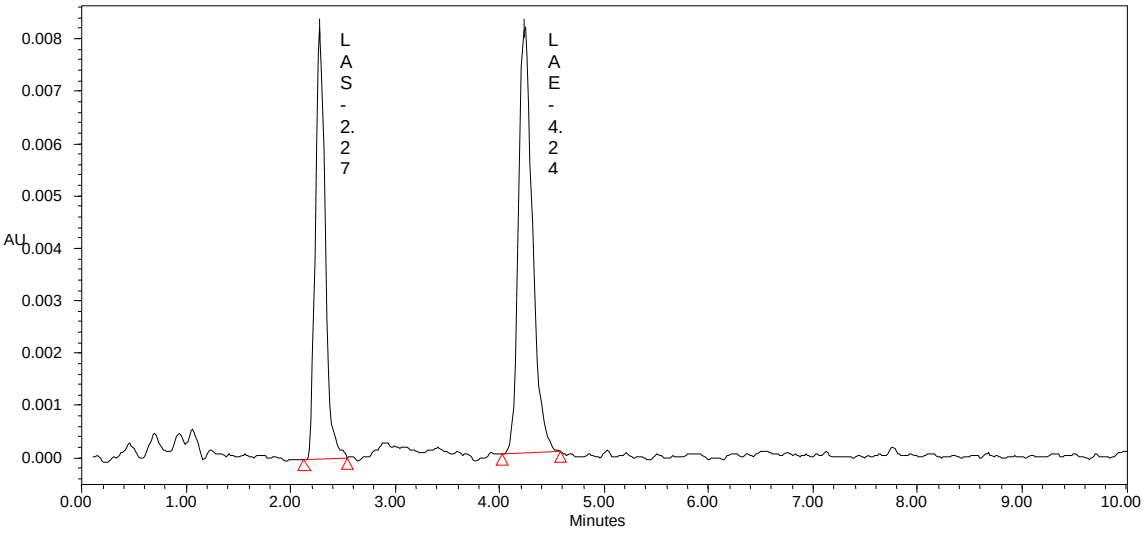
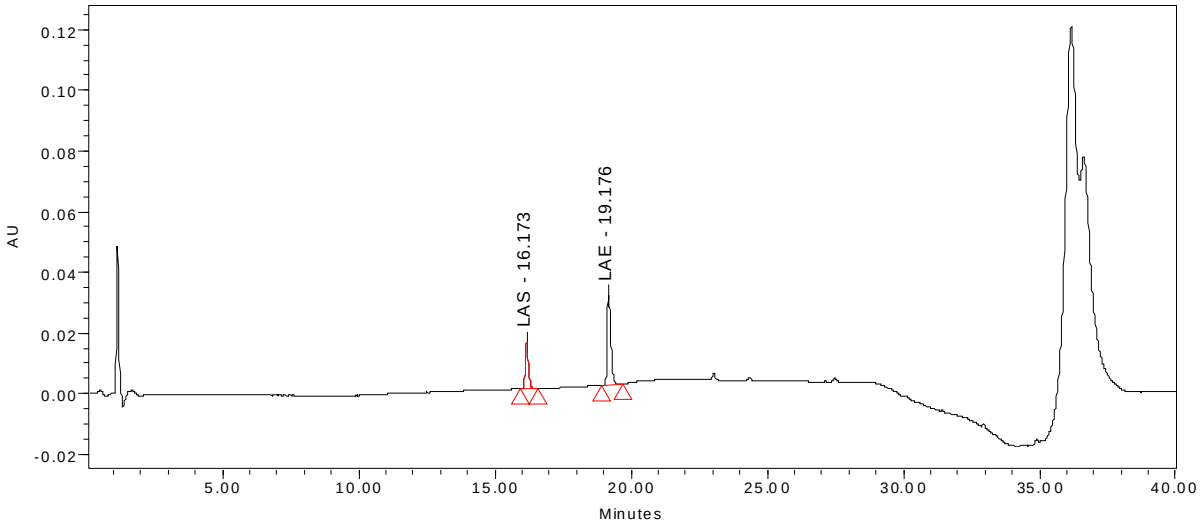
8. CHROMATOGRAPHIC PROFILES

Isocratic conditions



Gradient conditions



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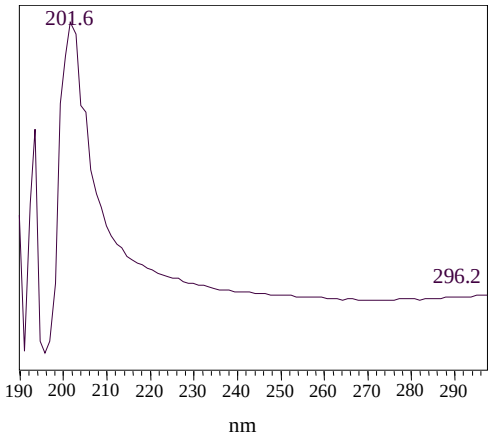
Ultraviolet profiles of LAS and ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl

LAS



Ethyl-N$^{\alpha}$-lauroyl-L-arginate HCl