Chapter 23

Ethyl Lauroyl Arginate (LAE): Antimicrobial Activity and Applications in Food Systems

C. Nerin, R. Becerril, S. Manso and F. Silva
University of Zaragoza, Zaragoza, Spain

Ethyl lauroyl arginate (Figure 1) (ethyl-N^{α}-dodecanoyl-L-arginate hydrochloride, LAE, CAS number 60372-77-2) is a cationic surfactant (Asker et al., 2009; Kawamura and Whitehouse, 2008) that was approved and generally recognized as safe (GRAS) for certain food applications within the United States by Food and Drug Administration. LAE was evaluated for food safety as an antimicrobial in food by FDA (2005), and as a food preservative by EFSA (2007). According to the Food and Agriculture Organization (FAO), the European Food Safety Authority, and the Food and Drug Administration (FDA), this substance can be referred to as ethyl lauroyl arginate HCl, lauric arginate ethyl ester, lauramide arginine ethyl ester, LAE, INS No. 243, EC No. 434-630-6, and E243 (EFSA, 2007; FDA, 2005; Kawamura and Whitehouse, 2008).

23.1 MANUFACTURING AND PHYSICAL-CHEMICAL PROPERTIES

Ethyl lauroyl arginate is a white hygroscopic powder, with a molecular weight of 421.02 g/mol that melts at temperatures in the range of 50.5-58.0 °C and decomposes at temperatures above 107 °C (FDA, 2005). Due to its polar properties, this compound has good water solubility (above 247 g/kg) and a low oil-water equilibrium partition coefficient ($K_{ow} < 0.1$), which means it tends to concentrate in the water phase of products (Asker et al., 2009; FDA, 2005). Besides being soluble in water, LAE is also freely soluble in ethanol, propylene glycol, and glycerol, yielding solutions with pH values ranging from 3.0 to 5.0 (1% solution) (Kawamura and Whitehouse, 2008). LAE is synthesized through L-arginine monohydrochloride esterification with ethanol using thionyl chloride as an esterification agent. The ester formed then condensates with lauroyl chloride in an aqueous medium to form LAE (Asker et al., 2009; Kawamura and Whitehouse, 2008). This production process was patented by the Spanish company LAMIRSA and includes the two aforementioned synthesis steps and a final filtration process to recover the product (FDA, 2005). This filtration step yields a food-grade product, consisting of an aqueous paste containing between 71% and 81% of the active ingredient, ethyl-N^{α}-lauroyl-L-arginate hydrochloride. When dried, this percentage increases to a 85-95% of the active ingredient (FDA, 2005). The food-grade specifications of dehydrated food-grade LAE are presented in Table 1.

LAE is stable for more than 1 year at 25 °C (room temperature) in a tight container protected from light. However, under severe conditions, such as very low pH (<1.5) and high temperature (more than 5 h at 100 °C and 1 h at 121 °C), and over extended periods of time, the product can suffer degradation (FDA, 2005). Notwithstanding, the resulting degradation products are the same nontoxic compounds listed (Table 1) as impurities or residuals in LAE, such as LAS, lauric acid, ethyl laurate, arginine, and arginine ethyl ester.

Regarding this compound's reactivity, it is known that LAE interacts with anionic components through electrostatic interactions. It tends to precipitate from solution at pH > 4.5 and at high ionic strength (Asker et al., 2009; FDA, 2005). This compound can also suffer hydrolysis in the presence of nitrites or proteins (Kawamura and Whitehouse, 2008).

23.2 METABOLISM AND TOXICOLOGICAL DATA ON LAE

Regarding its metabolism, LAE is hydrolyzed in the human body by chemical and metabolic pathways, which quickly break the molecule into its natural components, lauric acid and L-arginine, releasing ethanol and giving this compound

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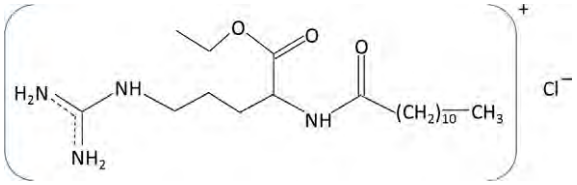

FIGURE 1 Chemical structure of LAE.

TABLE 1 Specifications of Food-Grade LAE According to the FDA (FDA, 2005)

Formulation: Components, Residuals, Contaminants		Percentage
Principal component	Ethyl-N^{α}-lauroyl-L-arginate HCl	85-95
Other related substances	N^{α}-lauroyl-L-arginine (LAS)	≤3
	Ethyl laurate	≤3
	Lauric acid	≤5
	Arginine HCl	≤1
	Ethyl arginine 2HCl	≤1
Residuals-volatiles, solvents, etc.	Ethanol	≤0.2
Ash		≤2
Water		≤5
Heavy metals, inorganics	Heavy metals (as lead)	≤10
	Arsenic (As)	≤3
	Lead (Pb)	≤1
	Cadmium (Cd)	≤1
	Mercury (Hg)	≤1

its known low toxicity (Asker et al., 2009). The L-arginine formed is further converted to ornithine and urea through the urea cycle. On its turn, L-ornithine can be further degraded to citrulline in the urea cycle or to α-ketoglutarate, a metabolic intermediate of the citric acid cycle, yielding CO_2 as final product. Therefore, the two major routes of LAE metabolites excretion is expired air, in the form of CO_2, and urine, in the form of urea (Figure 2) (EFSA, 2007).

In order to demonstrate LAE safety, several *in vitro* and *in vivo* metabolism—toxicokinetics studies; *in vitro* and *in vivo* mutagenicity assays; acute, sub-chronic, and chronic toxicity studies; reproduction and developmental toxicity studies (one and two generations) in animals; and human kinetic studies were performed according to the Organization for Economic Co-operation and Development (OECD) and Good Laboratory Practices (GLP) guidelines (Table 2). Although a 52-week chronic study is presented, there is a lack of long-term studies on LAE toxicity. Regarding acute dermal toxicity and irritation data, it was found that this compound is not a skin sensitizer as a single dermal application of 2000 mg/kg/day yielded no systemic effects, with dermal irritation in the application site only. The most relevant adverse effects were observed during the acute eye irritation test, where this compound was proven to be a severe eye irritant. Additionally, during the acute dermal toxicity test in rabbit-occluded skin, a mild dermal irritation was observed (Ruckman et al., 2004).

Potential dietary exposure to ethyl lauroyl arginate was estimated based on United Kingdom food consumption data, assuming that this compound would be present in all food categories with proposed use levels. Taking this information into consideration, the EFSA panel established an acceptable daily intake (ADI) for ethyl lauroyl arginate of 0.5 mg/kg bw.

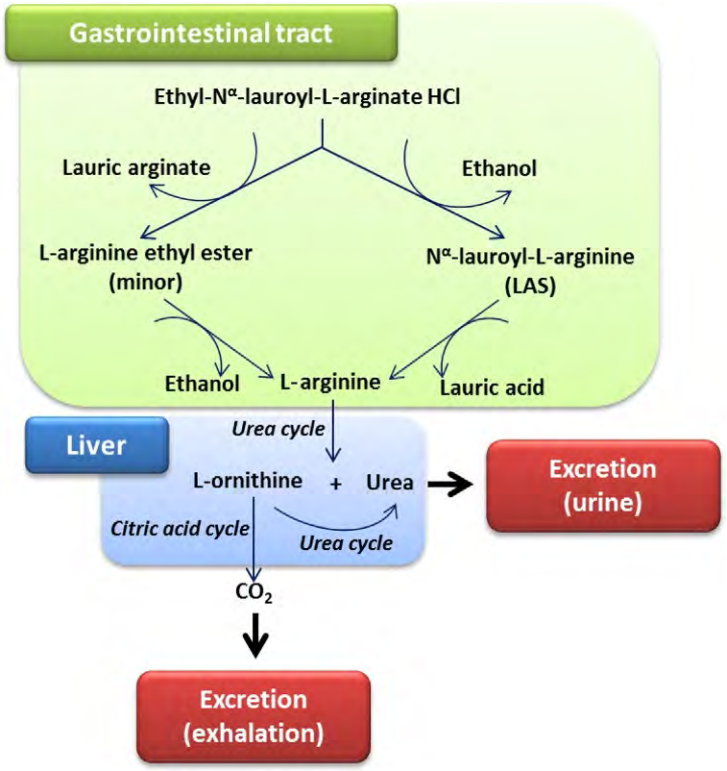

FIGURE 2 Biotransformation pathway of ethyl-*N*$^{\alpha}$-dodecanoyl-L-arginate hydrochloride in rats based on *in vitro* and *in vivo* data. *Adapted from EFSA (2007).*

TABLE 2 Overview of LAE Toxicological Studies

Toxicity	Compound/Solution	Toxicity Levels and Effects	Testing Subjects and Methods
Acute oral toxicity	Ethyl lauroyl arginate	LD_{50} higher than 2000 mg/kg bw	Male and female Sprague–Dawley rats
	19.5% solution of ethyl lauroyl arginate in propylene glycol		
	LAS		
Sub-chronic toxicity	Ethyl lauroyl arginate	No observed adverse effect level (NOAEL) 5000 mg/kg diet (384 and 445 mg/kg bw/day, for males and females respectively) in rats	13 week studies in male and female Han Wistar rats
	19.5% solution of ethyl lauroyl arginate in propylene glycol	NOAEL of 12800 mg/kg diet (904 and 1067 mg/kg bw/day for males and females, respectively) in rats	13 week studies in male and female Sprague–Dawley rats
Reproductive and developmental toxicity (one-generation studies)	Ethyl-*N*$^{\alpha}$-lauroyl-L-arginate	NOAEL for the females was 138 mg/kg bw/day	Pregnant rats with study end on gestation day 20
		NOAEL for the foetuses was of 1382 mg/kg bw/day	
		NOAEL of 207 mg/kg bw/day for females	Pregnant rabbits
		NOAEL of 691 mg/kg bw/day for foetuses	

Continued

TABLE 2 Overview of LAE Toxicological Studies—cont'd

Toxicity	Compound/Solution	Toxicity Levels and Effects	Testing Subjects and Methods
Reproductive and developmental toxicity (two-generation studies)	Ethyl-N^{α}-lauroyl-L-arginate)	NOAEL for reproductive performance 1073 mg/kg bw/day in adults	Sprague–Dawley adult rats and their offspring
		NOAEL of 2600 mg/kg bw/day for females during lactation	
Mutagenicity	Ethyl-N^{α}-lauroyl-L-arginate	Up to a dose of 150 µg/mL, no clastogenic activity was observed	*In vitro* cytogenetic test in human lymphocytes
	19.5% solution of ethyl lauroyl arginate in propylene glycol	No increase in mutation frequency	Mouse lymphoma cell mutation test
		Up to a dose of 500 µg/mL, no clastogenic activity was observed	Human lymphocytes cytogenetic test
	N^{α}-lauroyl-L-arginine (LAS)	No mutagenic activity up to concentrations of 5000 µg/plate	Ames test using *Salmonella* and *E. coli*
		After oral administration of 2000 mg/kg bw, no genotoxic effects were observed	Induction of micronuclei of polychromatic erythrocytes from mouse bone marrow
Chronic toxicity	ethyl lauroyl arginate (88.2% purity)	The study indicates a NOAEL of 307 mg/kg bw/day and 393 mg/kg bw/day for males and females, respectively	52 week chronic study in rats
		The EFSA panel concluded that NOAEL is lower than 106 mg/kg bw/day	
Human data		No clinically significant abnormalities in blood chemistry data, no notable changes in vital signs and no significant findings in ECG	Six healthy volunteers assigned to two dose group (2.5 and 1.5 mg/kg bw)

Data withdrawn from EFSA (2007) and Ruckman et al. (2004).

23.3 ANTIMICROBIAL ACTIVITY

According to LAMIRSA technical leaflet, ethyl lauroyl arginate has a strong antimicrobial activity against a wide range of microorganisms that include molds, yeasts, gram-negative, and gram-positive bacteria. However, the number of scientific papers dealing with antimicrobial LAE activity is scarce. Thus, the number of strains tested is limited and focused mainly on bacteria. Table 3 presents the microorganisms evaluated *in vitro* in scientific works and the values of MICs and MBCs or MFCs obtained.

According to these data, MICs obtained in literature (ranged from 8 to 100 ppm) are consistent with those mentioned in the technical leaflet of LAE (ranged from 2 to 128 ppm), indicating a strong antimicrobial activity of LAE.

Since LAE may be used as an antimicrobial agent in food packaging, it is relevant to assess how long it takes for this compound to be effective. With this purpose, different works have studied the bactericidal or bacteriostatic effect of LAE by kill-time analysis, plotting the number of viable cells after treatment with LAE against time. Although methodology used in the different works may diverge in inoculum size or LAE concentration, all works concluded that LAE has a fast bactericidal action. Pattanayaiying et al. (2014), Becerril et al. (2013), and Soni et al. (2010) observed the reduction of bacterial population in at least 3.5 log CFU/mL in less than 1 h for *L. monocytogenes*, *L. innocua*, *B. thermosphacta*, *S. enterica*, *E. coli*, *S. aureus*, and *P. aeruginosa*. Some authors have observed a slower bactericidal effect on *L. monocytogenes*, *S.* Rissen, and *E. coli* O157:H7 with a reduction of approximately 4 log CFU/mL in 3, 6, and 4 h respectively (Suksathit and Tangwatcharin, 2013; Pattanayaiying et al., 2014).

TABLE 3 MICs and MBC or MFC values in μg/g (ppm)

Microorganism	MIC	MBC (or MFC)	Reference
Salmonella enterica	32 ppm		(Rodríguez et al., 2004)
	16 ppm	32 ppm	(Suksathit and Tangwatcharin, 2013)
	25 ppm	25 ppm	(Becerril et al., 2013)
	23.5 ppm	23.5 ppm	(Ma et al., 2013)
Escherichia coli	25 ppm	25 ppm	(Becerril et al., 2013)
	25 ppm	25 ppm	(Otero et al., 2014)
	11.8 ppm	11.8 ppm	(Ma et al., 2013)
Pseudomonas aeruginosa	100 ppm	100 ppm	(Becerril et al., 2013)
Listeria innocua	25 ppm	25 ppm	(Becerril et al., 2013)
Listeria monocytogenes	8 ppm	32 ppm	(Soni et al., 2010)
	≤25 ppm		(Suksathit and Tangwatcharin, 2013)
	11.8 ppm	23.5 ppm	(Ma et al., 2013)
Staphylococcus aureus	8 ppm		(Rodríguez et al., 2004)
	12.5 ppm	50 ppm	(Becerril et al., 2013)
Aspergillus flavus	100 ppm	200 ppm	(Manso et al., 2010)

Using a different approach, Shen et al. (2015) studied the bactericidal effect of LAE after adaptation of *L. monocytogenes* to acidic media. The study resulted in an increase of resistance to LAE since acidic-adapted cells survival was approximately 2 log CFU/mL greater than nonadapted cells.

23.3.1 Antimicrobial Mechanism of Action

The main target of cationic surfactants is the cell envelope. Due to their chemical structure, these compounds can damage bacteria membranes, producing alteration of membrane potential and membrane permeability. This results in the loss of cytoplasmatic material and, consequently, bacterial death (Rodríguez et al., 2004).

As a cationic surfactant, LAE is expected to show a similar mechanism of action. For this reason, investigations about its mode of action are focused on the study of morphology changes by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) and in the study of the cell envelope integrity (flow cytometry and ion efflux). Table 4 presents the different assays carried out to investigate the mode of action of LAE and the results obtained. Overall, the data described on the several assays dealing with ethyl lauroyl arginate antimicrobial action highlights that the cell envelope is its main target on bacteria.

On one hand, electron microscopy (SEM and TEM) shows cell envelope alterations like irregular shape, rough surface, or swelling. Notwithstanding, the same effects are not described in all bacteria. For example, abnormal septation was only observed in *S. aureus* and *L. monocytogenes* (Pattanayaiying et al., 2014; Rodríguez et al., 2004). The cell envelope damages are further confirmed by flow cytometry that indicated damage in cell membranes of *S. thyphimurium* and *S. aureus* (Rodríguez et al., 2004). In this case, different levels of damage were detected, depending on the bacteria. In *S. aureus*, for example, high amounts of partially damaged cell were also observed.

The results obtained for the efflux assays also confirmed the membrane damages caused by LAE. Intracellular potassium leakage and decrease of the proton flux, which are closely related to alterations in membrane permeability, were observed (Pattanayaiying et al., 2014; Rodríguez et al., 2004).

TABLE 4 Techniques Used to Study the Effects of LAE in Bacteria

Technique	Bacteria	Main Effects	Reference
Transmission electron microscopy (TEM) and/or scanning electron microscopy (SEM)	*Salmonella thyphimurium*	Outer membrane swelling; membrane-enclosed vacuoles; cell and cytoplasm integrity	Rodríguez et al. (2004)
	Staphylococcus aureus	Mesosome-like formations; intracytoplasmic white spots and clear zones; multiseptated cell; cell integrity	
	Escherichia coli O157:H7	Intracytoplasmic coagulation; distorted and dimpled cells	Pattanayaiying et al. (2014)
	Listeria monocytogenes Scott A	Intracytoplasmic coagulation; abnormal septation; irregular cross-wall formation; altered cell morphology; cell integrity	
	Brochothrix thermosphacta	Intracytoplasmic coagulation, pleomorphic, distorted and dimpled cells; cell integrity	
	Listeria monocytogenes and *Salmonella* Rissen	Membrane leakage; changes in cell envelope	Suksathit and Tangwatcharin (2013)
	Escherichia coli	Irregular shape; rough surface; pore formation; cell debris	Becerril et al. (2013)
Staining and flow cytometry	*Salmonella thyphimurium* and *Staphylococcus aureus*	Damaged cell membranes	Rodríguez et al. (2004)
Ion efflux study	*Salmonella thyphimurium* and *Staphylococcus aureus*	Intracellular potassium leakage and decrease of the proton flux	Rodríguez et al. (2004)
	Escherichia coli O157:H7, *Listeria monocytogenes Scott A* and *Brochothrix thermosphacta*	Intracellular potassium leakage	Pattanayaiying et al. (2014)

23.4 THE ROLE OF LAE IN FOOD SYSTEMS

As we have already seen, due to the lack of volatility, LAE needs a direct contact with the food product in order to be efficient. Nevertheless, LAE has been described as an interesting alternative compared with other antimicrobials such as essential oils, because LAE is an odorless compound of great potential (Becerril et al., 2013). In addition, LAE could be used in combination with other food antimicrobials in lower concentrations, due to the possibility of a synergistic effect. For example, Ma et al. (2013) observed synergism between LAE and cinnamon or eugenol in *L. monocytogenes*, but antagonism in *E. coli* and *S.* Enteritidis. Suksathit and Tangwatcharin (2013) described an enhancing antimicrobial effect when combining LAE with organic acid salts (sodium diacetate, sodium citrate, and sodium lactate) against *S.* Rissen and *L. monocytogenes*. Pattanayaiying et al. (2014) reported an antimicrobial effect of nisin Z in combination with LAE on gram-negative bacteria.

Besides, as it has been previously discussed, the strong activity shown in *in vitro* assays has led not only to the direct application to the external surface of a food product, but also to the incorporation into different packaging materials (Table 5). In fact, LAE may even be used in active packaging that requires high temperature technologies since it has been demonstrated than LAE can maintain its antimicrobial activity against *E. coli* after heat treatment (90 or 120 °C for 15 min) (Becerril et al., 2013).

However, as in other antimicrobials, the composition of the matrix influences the activity of the antimicrobial compound. Hence, many authors have obtained a much higher activity of LAE when tested *in vitro*, compared with the *in vivo* samples (Nair et al., 2014; Oladunjoye et al., 2013; Otero et al., 2014; Sharma et al., 2013). Continuing with this approach,

TABLE 5 Examples of Direct Addition of LAE on the Surface of Several Products

Application	Target	Results	Reference
Direct addition	Apple juice samples contaminated with *E. coli* 0157:H7	The maximum bacterial reduction with PEF treatment was found between 3 and 4 log cycles, whereas with the addition of 50 ppm of LAE the activity was increased up to 6 log cycles, reducing also the treatment time	Saldaña et al. (2011)
Direct addition	Pasteurized 3.25% fat chocolate or unflavored milk products	The addition of 200 mg/mL LAE was more effective in unflavored milk samples, giving at 21 day a reduction of 5.77 log CFU/mL, compared to the 0.9 log CFU/mL decrease of the chocolate samples	Woodcock et al. (2009)
Direct addition	2% reduced fat milk inoculated with *L. monocytogenes*, *S.* Enteritidis, and *E. coli* 0157:H7	The antilisterial activity was achieved with 6000 ppm of cinnamon essential oil, eugenol or thymol, or from 750 ppm LAE in 2% reduced fat milk. Besides, the growth curves showed great differences depending on the strain. Hence, the combination of LAE and EO gave the most effective result against *L. monocytogenes*, whereas in the other strains the best result was obtained with the EO alone	Ma et al. (2013)
Surface treatment	Chicken breast fillets inoculated with *C. jejuni*	Despite the strong activity *in vitro* experiments, LAE treatment at 200 and 400 mg/kg was only effective in the chicken breast fillets inoculated with *C. jejuni*, whereas it was not effective against mesophilic growth and it had a very low activity in the case of psychrotrophs	Nair et al. (2014)
Surface treatment	Skinless, boneless chicken breast fillets inoculated with *Salmonella*	200 and 400 ppm of LAE did not reduce the mesophilic counts of the chicken samples, but it was effective against *Salmonella* inoculation, decreasing about 1 log CFU/g. Besides, LAE did not modify the pH of the chicken breast fillets	Sharma et al. (2013)
Surface treatment	Frankfurters inoculated with *L. monocytogenes*	The best results were obtained in frankfurters formulated with lactate/diacetate and treated with LAE (22, 27.5, and 33 ppm/454 g frankfurters) into the packaging, keeping the *L. monocytogenes* growth below 2 log CFU/cm^2 through the shelf life of 156 days of refrigerated storage	Martin et al. (2009)

Ma et al. (2013) demonstrated that the addition of soluble starch increased, in a high manner, the minimum bactericidal concentration of LAE against *Listeria monocytogenes*. Considering the influence of fat content, Sharma et al. (2013) obtained a much higher LAE activity in the case of skinless chicken breast fillet, than in ground chicken containing a higher fat concentration. Despite this, the authors Oladunjoye et al. (2013) did not find a relationship between the LAE activity and the fat content in the case of ground turkey samples.

Similarly to other antimicrobial agents, LAE has the possibility of being directly added into the food sample or being incorporated into an active packaging. In the latter, it is important to point out the relevance of the polymer, which exerts an essential role on the diffusion rate of the active compound, and, therefore, on the final bioavailability of the antimicrobial agent applied (Luchansky et al., 2005; Muriel-Galet et al., 2015; Taormina and Dorsa, 2009).

Many authors have highlighted that, despite the fact that LAE has been demonstrated to possess a much lower activity when used in real food products compared with the *in vitro* studies, LAE is an interesting alternative when used in combination with other technical procedures. In this context, the authors Saldaña et al. (2011) demonstrated that the addition of LAE at 25 and 50 ppm together with the treatment of pulsed electric fields caused a higher reduction of *E. coli.* while reducing the cost of the physical treatment. Similarly, in another work, the best antimicrobial results were obtained when combining LAE with other antimicrobials such as carvacrol (Oladunjoye et al., 2013).

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