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Mass spectrometric studies of ionic reactions in confined volumes

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1. Introduction

Mass spectrometry is one of the most widespread analytical techniques applied to molecules recognition.

Despite its analytical application, is it possible to use this technique for purposes other than molecule recognition?

The answer is definitely yes: mass spectrometry not only recognizes molecules but can also synthesize them under ambient conditions through reactions which are often more efficient than the same processes performed in bulk.

The use of confined volumes in chemical reactions introduces unique conditions, such as rapid solvent evaporation, increased reactant concentrations, and elevated surface-to-volume ratios, which significantly accelerated reaction kinetics compared to bulk methods.

This development was possible thanks to the introduction of the electrospray ionization (ESI) source by Fenn in 2002 that was awarded with the Nobel Prize.

Actually, the ESI source is the most widely used mass spectrometric ionization technique and can be applied to the analysis of a large number of inorganic and organic compounds as well as biological molecules.

In the ESI source a strong electric field (kV) converts a liquid spray into a charged microdroplet aerosol.

In these processes, the high electric field forms a droplet jet from a Taylor cone (Figure 1).

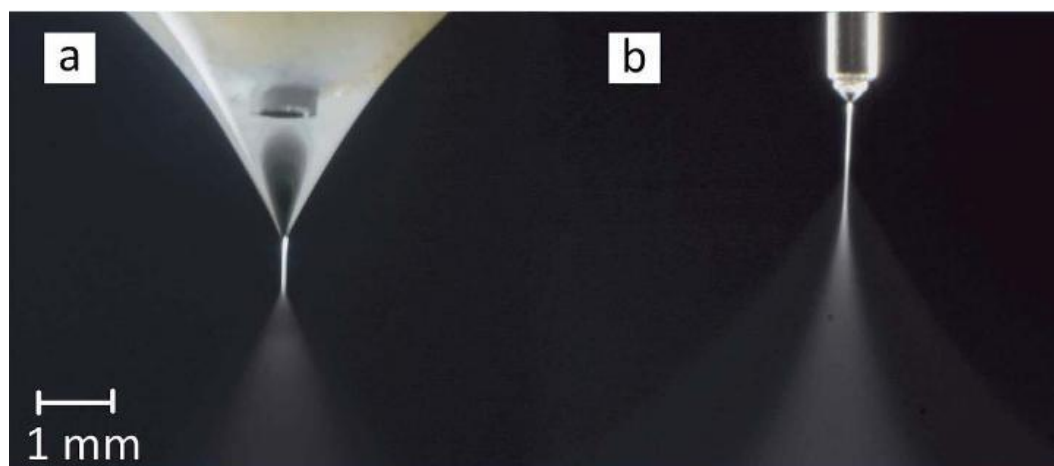


Figure 1. The figure shows a Taylor cone. When the applied potential is high enough that the electrostatic repulsive forces on the droplet exceed the surface tension, the droplet is gradually deformed until the so-called "Taylor cone" is formed. Photos made by Sereshkeh et al. [1].

The coulombic explosion of the microdroplets due to the strong electric field leads to the almost complete desolvation of the polar compounds contained within them, producing ions that enter in the vacuum region of the mass spectrometer through a micrometer aperture to be mass separated by the analyser (Figure 2).



Figure 2. *Electrospray Ionization source of a Thermo Fisher mass spectrometer.*

Recently, the ESI source was used to synthetic purposes thanks to the intuition of R.G. Cooks and R.N. Zare.

In fact, in their first studies published about ten years ago, they demonstrated that ionic reactions can be induced and greatly accelerated in the ESI charged microdroplets generated by the electrospray ionization process respect to the same reaction in bulk.

The scientific community has observed that when the reactants are confined in the ESI microdroplets, various fundamental reactions are accelerated by several orders of magnitude compared to the same reaction in bulk (Table 1) [2].

Table 1. Reaction classes, general reaction schemes, and corresponding acceleration methods. Abbreviations: DESI (desorption electrospray ionization); ESI (electrospray ionization); nESI (nanoelectrospray ionization) Cooks et al. [2].

Classification	Reactions	Chemical equations	Methods (reference)
1. Addition	1.1. Michael or aza-Michael addition	$\begin{array}{c} R^1 \\ \\ YH \\ \\ R^2 \end{array} + \begin{array}{c} R^4 \\ \backslash \\ C \\ / \\ R^3 \end{array} \longrightarrow \begin{array}{c} R^1 \\ \\ R^2 - Y - CH - R^4 \\ \\ R^3 \end{array}$ $R^2 = \text{Electron withdrawing group (EWG)}$ $Y = CH, R^1, R^2 = \text{EWG or } Y = N$	Reactive DESI (27) Dropcast thin film (29)
2. Elimination	2.1. Dehydration	$\begin{array}{c} R^1 \\ \\ R^2 - C - C - OH \\ \quad \\ R^3 \quad R^4 \end{array} \longrightarrow \begin{array}{c} R^1 \\ \\ R^2 - C = C - R^4 \\ \\ R^3 \end{array} + H_2O$	Leidenfrost droplet (50)
3. Addition and elimination	3.1. Schiff base formation	$R^1 - NH_2 + \begin{array}{c} O \\ \\ R^2 - C - R^3 \end{array} \longrightarrow \begin{array}{c} R^1 \\ \\ N \\ \\ R^2 - C - R^3 \end{array} + H_2O$	ESI (40) Reactive DESI (27) Dropcast thin film (36) Leidenfrost droplet (48)
	3.2. Claisen-Schmidt condensation	$\begin{array}{c} O \\ \\ R^1 - C - R^2 \end{array} + \begin{array}{c} O \\ \\ R^3 - C - R^4 \end{array} \longrightarrow \begin{array}{c} O \\ \\ R^1 - C - C - R^4 \\ \quad \\ R^2 \quad R^3 \end{array} + H_2O$	Preparative ESI (28) Leidenfrost droplet (48) Thin film (31, 33, 34)
	3.3. Katritzky transamination reaction	$R^1 - NH_2 + \begin{array}{c} R^3 \\ \\ R^2 - C - C - R^4 \\ \quad \\ O \quad O \end{array} \longrightarrow \begin{array}{c} R^3 \\ \\ R^2 - C - C - R^4 \\ \quad \\ N \quad O \end{array} + H_2O$	Leidenfrost droplet (50) Thin film (30, 34)
	3.4. Amide formation	$\begin{array}{c} R^1 \\ \\ NH \\ \\ R^2 \end{array} + \begin{array}{c} O \\ \\ X - C - R^2 \end{array} \longrightarrow \begin{array}{c} R^1 \\ \\ N - C - R^2 \\ \\ R^2 \end{array} + H-X$ $X = \text{Leaving group}$	nESI (81) ESI (82)
	3.5. Hydrolysis of esters	$H_2O + \begin{array}{c} O \\ \\ R^1 - C - O - R^2 \end{array} \longrightarrow \begin{array}{c} O \\ \\ R^1 - C - OH \end{array} + H_2O - R^2$	ESI (72)
4. Oxidation and reduction	4.1. Oxidation of amine or sulfide	$H_2O_2 + \begin{array}{c} R^3 \\ \\ R^1 - N - R^2 \\ \\ O \end{array} \longrightarrow \begin{array}{c} R^3 \\ \\ R^1 - N^+ - R^2 \\ \\ O^- \end{array} + H_2O$	Levitated droplet (50)
	4.2. Synthesis of nanoparticles	$H_2O_2 + R^1 - S - R^2 \longrightarrow \begin{array}{c} O \\ \\ R^1 - S - R^2 \end{array} + H_2O$	Levitated droplet (83) ESI (84)
5. Multi-component reaction	5.1. Mannich condensation	$\begin{array}{c} R^1 \\ \\ NH \\ \\ R^2 \end{array} + \begin{array}{c} O \\ \\ H - C - R^3 \end{array} + \begin{array}{c} R^4 \\ \\ R^5 - C - R^6 \\ \\ O \end{array} \longrightarrow \begin{array}{c} R^1 \\ \\ N - C - C - R^6 \\ \quad \\ R^2 \quad R^5 \end{array}$	Thin film (30, 34)
	5.2. Hantzsch reaction	$\begin{array}{c} O \\ \\ R^1 - C - C - O - R^2 \end{array} + \begin{array}{c} O \\ \\ H - C - R^3 \end{array} + NH_3 \xrightarrow{[O]} \begin{array}{c} R^2OOC \\ \\ R^1 - C - C - COOR^2 \\ \quad \\ R^3 \quad R^1 \end{array}$	ESI (37)
	5.3. Biginelli reaction	$\begin{array}{c} O \\ \\ R^1 - C - C - O - R^2 \end{array} + \begin{array}{c} O \\ \\ H - C - R^3 \end{array} + H_2N - C(=O) - NH_2 \longrightarrow \begin{array}{c} R^2OOC \\ \\ R^1 - C - C - NH \\ \quad \\ R^3 \quad R^1 \end{array}$	nESI (85)
6. Others	6.1. Hydrogen-deuterium exchange	$R-H \xrightarrow{D_2O} R-D$	Droplet fusion (46) nESI (86)
	6.2. Protein folding and unfolding	$* \cdots * \longrightarrow *$	nESI (87) Droplet fusion (46)
	6.3. Organometallic reactions	$\begin{array}{c} \text{Pyridine-I} \\ + R-OH \end{array} \xrightarrow{[Cu]} \begin{array}{c} \text{Pyridine-OR} \end{array}$ $\begin{array}{c} \text{Pyridine-X} \\ + \text{HO-B(OH)-Ph-OH} \end{array} \xrightarrow{[Pd]} \begin{array}{c} \text{Pyridine-Ph-OH} \end{array}$ $X = Cl, Br, I$	ESI and thin film (35) Levitated microdroplet (88)
	6.4. Pinacol rearrangement	$HO - C(CH_3)_2 - C(CH_3)_2 - OH \longrightarrow (CH_3)_3C - C(=O) - CH_3$	ESI (63)

Among recent noteworthy examples in this research field there is the Zare's study about synthesis of urea in microdroplets [3].

Urea $\text{CO}(\text{NH}_2)_2$ was the first molecule to be successfully synthesized in the laboratory.

It is produced in excess of 190 million tonnes per year as it is used both as a fertiliser and as an additive in biodiesel.

In bulk, the reaction is complex and requires high-energy sources: for example, temperatures of up to 500°C and pressures of 250 bar.

There are no other more environmentally friendly synthetic methods.

However, Zare et al. recently proposed a simpler and greener synthetic mechanism by exploiting the properties of aqueous microdroplets.

In fact, they were able to create a three-phase (gas-liquid-solid) heterogeneous catalytic system using N_2 and atmospheric CO_2 in contact with a graphite network catalyst surface coated with CuBi_2O_4 .

In a single step and in fractions of a second, without the use of high temperatures (ambient temperature), they were able to convert CO_2 into urea.

Another recent study by Zare et al. demonstrated that the formation of hydroxyl radicals on the water-air surface of aqueous microdroplets [4] is capable to canalize the formation of anilines from carboxylic acids without the use of metal catalysts or organic solvents.

The hydroxylamine attacks the protonated benzoic acid, leading to the formation of the final product through decarboxylation.

This is a further environmentally friendly method for the synthesis of anilines and N-substituted anilines.

The peculiarities of aqueous microdroplets were also exploited by R.G. Cooks for the synthesis of proteins.

The formation of peptides from amino acids can take place at the air/water microdroplet interface [5].

The amino acid complex in the microdroplets undergoes dehydration to form oxazolone, the addition of water to oxazolone forms the dipeptide and the reaction of oxazolone with other amino acids forms tripeptides.

Furthermore, the chirality of the amino acids involved is preserved in the oxazolone product, and strong chiral selectivity is observed in the conversion of oxazolone to tripeptide.

The results obtained suggest a role for oxazolone in the prebiotic synthesis of peptides, providing a possible mechanism as to how peptides could have been formed in the Earth's primordial conditions.

This method of synthesis ensures that even optically impure amino acids can elongate the chain, generating pure, homochiral peptides.

These examples demonstrate the power of performing ionic reactions in microdroplets as a new synthetic method and the scientific community is laying the foundations for its large-scale usage in the future.

1.1 ESI Source Parameters and Factor Influencing Microdroplets Reaction Kinetics

ESI parameters such as the distance between the spray source and the MS inlet must be carefully defined as they play an important role in controlling the microdroplets reaction kinetics.

Microdroplet size and lifetime depends to the emitter tip diameter and to the distance of the electro spray emitter to the MS inlet.

Microdroplets reaction time is on the scale of microseconds to milliseconds, calculated as the distance divided by the velocity of the microdroplet jet, which varies with the distance they travel.

Several studies show an increasing reaction rate when the distance between the electrospray emitter and MS entrance was increased and when the sprayer diameter is reduced to decrease the droplet size and increase their surface to volume ratio.

Other experimental variables such as the solution flow rate, nebulizer gas pressure and the applied potential, control the nature of the initially formed droplets (e.g., size, solute velocity and distribution, nature and charge distribution) and this in turn determines the fate of the droplet and its loaded reactants, including molecular desolvation and ejection events.

A key factor that determines whether conditions favour reaction acceleration over reaction monitoring inside the ESI source is the nature of the desolvation process.

Continuous solvent evaporation from droplets, that individually contain molecules of both reagents should favour the accelerated reaction because

of the increased concentrations of the analytes and the pH changes that occur with evaporation.

The reaction in microdroplets, once the droplet reaches the vacuum of the ion source, will either stop at whatever stage it has reached.

Continuous desolvation favours reaction acceleration whereas discontinuity freezes out the system in its contemporaneous state without contributing to reaction acceleration.

The major factors that contribute to the acceleration of the reaction in the microdroplets are: i) the fast evaporation of the solvent with the associated increase in reactant concentrations, ii) the increased acidity or basicity inside the microdroplets, and iii) the confinement of the reactants in small volume reactors with high surface area to volume ratios.

The rapid and continuous evaporation of the solvent in the microdroplet increases the concentration of the reagent, leading to an increase in the reaction rate due to the enhancement of intermolecular collisions.

The evaporation of the solvent affects the size of the droplet; the more solvent evaporates, the smaller the droplet's diameter becomes.

The intrinsic factor believed to cause an increase in reaction rate in microdroplets is the role of the gas-liquid interface. For the same volume of solution, smaller droplets have proportionally more surface sites.

Even weak interactions of a reagent with the droplet interface can have powerful effects on reactivity.

Positioning of the reagent at the interface leads to faster reactions due to the reduced desolvation energy of the reagent with an incomplete solvent shell.

It is known that the reason why ion-molecule reactions in the gas phase have potential energy surfaces so different from those of conventional reactions in solution lies in the energetics of solvation. In bulk solution, large desolvation energy barriers must be overcome for reactions to occur, significantly slowing down the reactions rate and yields. The molecules residing on the surface of the droplets are only partially solvated and partially exposed to air.

The reactions on the surface of small droplets are similar to the reactions of naked gas-phase ions.

This means that molecules can more easily overcome the barriers associated with the activation energy of the reaction. For a generic reaction

between two generic reactants A and B to form generic products C and D, the reaction rate can vary depending on the reaction environment, as shown in Figure 3.

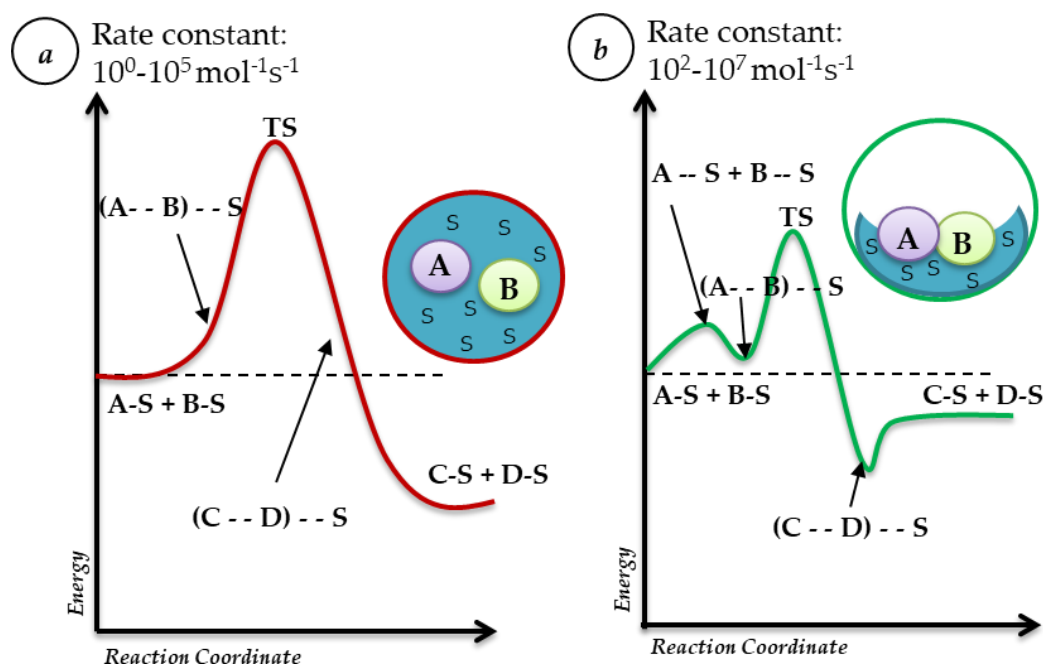


Figure 3. Comparison of the kinetic trend of the reaction in bulk (a) and in the microdroplets (b).

The complete solvation (S) of reactants in solution represents a significant barrier to overcome, which is why they often do not have high rate constants (Figure 3a).

Microdroplets, on the other hand, represent a simplified system (Figure 3b): the reactants need to overcome only a small solvation energy to reach the transition state (TS) due to partial solvation.

The degree of solvation influences the rate constants because, in the condensed phase, the presence of ion pairs and interactions with the solvent mask the intrinsic reactivity of the reactants in the reaction.

The ion/molecule reactivity of partially solvated ions decreases with the increase in the size of the solvation shell.

In other words, the decrease in the number of hydration shells of an ion will have a dramatic effect on the activation energy. If desolvation is the

only contributor to the activation energy, then a semi-solvated ion at an interface would benefit from a significant acceleration factor compared to the reaction in bulk phase, further simplifying the assumption of equal pre-exponential factors in the ratio of unimolecular rate equations.

When the solvent evaporates, most of the droplet surface tends to be occupied by surfactant analyte molecules, further decreasing the solvation energy because there would be fewer solvent molecules present.

Moreover, the evaporation of microdroplets can result in extreme pH values.

The pH value in the charged microdroplet can be varied by droplet evaporation but also by electrolytic redox reaction of water leading to the formation of H_3O^+ or OH^- ions.

Electrochemically induced changes in the solution composition can occur during the ESI process under certain conditions and with specific electroactive compounds.

Not only do these factors qualitatively explain the accelerated rate of reactions in small volumes, but the observed reaction kinetics also provide a basis for inferences about the positions of different analytes in microdroplets.

It should also be noted that the electric fields on highly curved interfaces (small droplets) are much greater than those on planar or weakly curved surfaces (large droplets).

A study by Zare et al. [6] described the ordered orientation of solute molecules within and near microdroplets using the anisotropy of fluorescence polarization.

The increase in concentration density at the interface is due to electrostatic repulsion, resulting in an ordered orientation of the reactant molecules: entropy decreases, and the free energy of the initial state increases.

It is known that lower entropy and, thus, higher free energy is associated with droplet reaction due to more ordered assembly on the droplet surface than inside the bulk.

Moreover, it is known that the reaction free energy in the gas phase is different from the reactions in solution and, thus, some reactions that cannot be achieved in the bulk can be achieved in a microdroplet due to its large interface between the solution and the gas phase.

When the droplet solvent evaporates, the density of reactant molecules at or near the droplet surface increases, making the reaction to occur on the gas-phase-like surfaces in terms of activation energy.

Solvent evaporation causes surface tension gradients, with the flow going from regions of low surface tension to those of high surface tension.

This effect is called the "Marangoni effect".

In a water-alcohol system, where alcohols exhibit lower surface tension and a lower boiling point, the evaporation of alcohol will be faster, increasing the surface tension on the microdroplet and causing the Marangoni flow.

The kinetics of a reversible reaction can be measured directly without the influence of the reverse reaction (*kinetic control*).

However, as time progresses, the contribution of the reverse reaction increases, and the rate gradually approaches zero as equilibrium is reached (*thermodynamic control*).

Partial solvation explains why reactions occurring in the surface region are accelerated and reach very high rate constants.

In contrast, within the droplet, conditions are comparable to those in solution. If the surface reaction is faster than the reaction inside the microdroplet, the neutral products return to the centre of the micro droplet, and other ionic reagents move to the surface region.

The concentration gradient is responsible for the flow of reagents until equilibrium is reached.

A study by Baret [7] describes an interfacial reaction-diffusion model useful for describing the influence of diffusion on reactions in confined volumes. They considered parallel reactions both in the surface region and the internal fraction, with diffusion occurring according to Fick's second law.

In the surface region, reagents orient themselves in an orderly fashion and form products that, in turn, redistribute throughout the droplet.

Other factors influencing the kinetics of the reactions in microdroplets described by Prophet et al. are [8]:

1. Kinetic Framework

A droplet consists of two kinetically distinct regions: the interface and the bulk interior. The surface region is approximately 1 nm thick, aligning with solvation energy profiles from molecular dynamics simulations.

This finite thickness allows for the definition of surface-adsorbed species using volumetric concentrations. The droplet's geometry is modelled as a rectangular prism, which replicates the surface-to-bulk scaling of a sphere. In reaction-diffusion simulations, the framework captures finite size effects, where molecules diffuse between compartments and undergo elementary reactions. At the interface, reactants A and B partition between the gas, interface, and bulk phases, described using rate constants (k_{solv} , k_{desolv} , k_{ads} , k_{des}) within a Langmuir framework. These constants establish characteristic lengths and timescales, leading to the notion of kinetic confinement. While the model simplifies by not resolving chemical gradients, it effectively approximates average concentrations of reactants A, B, and C, providing insights into kinetic changes when chemistry shifts from macro to microscale.

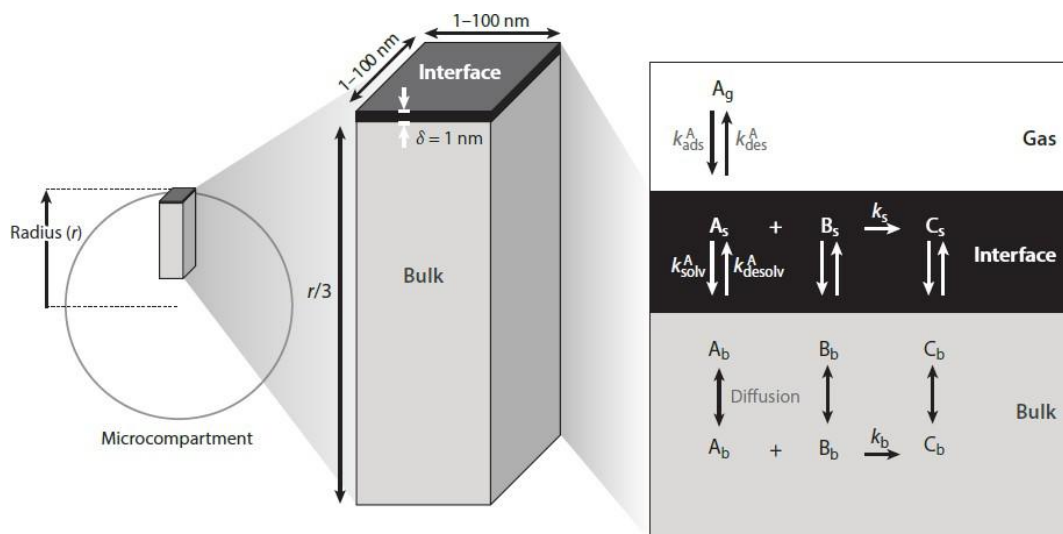


Figure 4. This conceptual framework describes reaction kinetics in microdroplets, simplified into two kinetically active regions connected by diffusion. The surface partitioning of reagents from the gas and liquid phases is characterized by a set of rate constants for adsorption, desorption, solvation, and desolvation.

The reactions $A + B \rightleftharpoons C$ occur both at the surface and in the bulk regions of the droplet. The interface is assumed to be 1 nm thick, with the height of the rectangular prism set at $r/3$ to mimic the surface-to-volume scaling of a sphere.

2. Kinetic Confinement

Unlike nanoconfinement, which pertains to molecular-scale dimensions, kinetic confinement occurs when the droplet size approaches specific lengths associated with chemical reactions, such as the reacto-diffusive (L_{rxn}) and adsorption (L_{ads}) lengths, along with the critical radius (r_{crit}).

These lengths are influenced by factors such as concentration, reaction rates, and diffusion coefficients (Figure 5).

The rate constants governing the movement of solutes to and from the interface (k_{solv} and k_{desolv}) play a key role in defining these lengths.

The relationship between the droplet size and these lengths determines whether the chemistry inside the droplet will differ significantly from that observed in larger-scale reactors.

Additionally, the width of the electric double layer in aqueous droplets, relative to droplet size, is another important characteristic length.

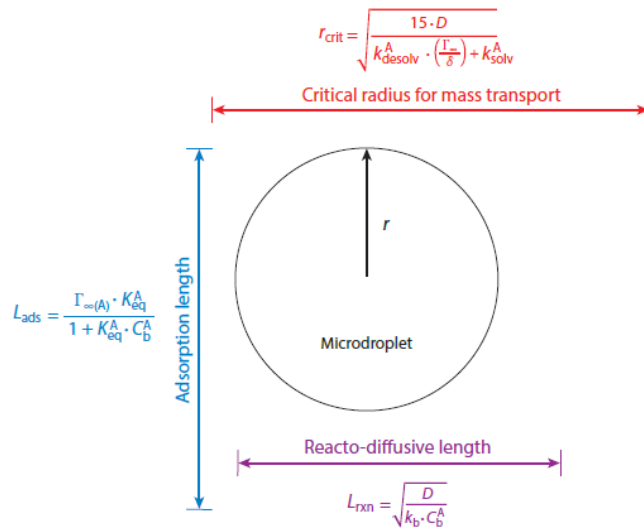


Figure 5. Characteristic lengths related to droplet size. C_b denotes the bulk concentration of solute A, while r represents the radius.

3. Fraction of Surface to bulk molecules

The parameter $N_{s/b}$ indicates the ratio of molecules at the droplet interface to those in the bulk interior, with significant differences between micro- and macroscale droplets shown in Figure 6.

$N_{s/b}$ is calculated from the number of surface (n_s) and bulk (n_b) molecules, with surface and bulk solute concentrations represented as C_s and C_b . In microdroplets, a larger fraction of molecules (0.1 to 10^2) reside at the interface, compared to a small fraction (10^{-3} to 10^{-5}) in macroscale containers.

While a higher $N_{s/b}$ does not automatically increase reaction rates, it can enhance sensitivity to differences between surface and bulk environments in microdroplets.

This parameter serves as an interface weighting factor, influencing chemical equilibria and reaction outcomes. Techniques like sum frequency generation and SHG can effectively probe interfacial dynamics, suggesting that creating microdroplets from bulk solutions is a valuable method for studying interface effects using less sensitive detection methods.

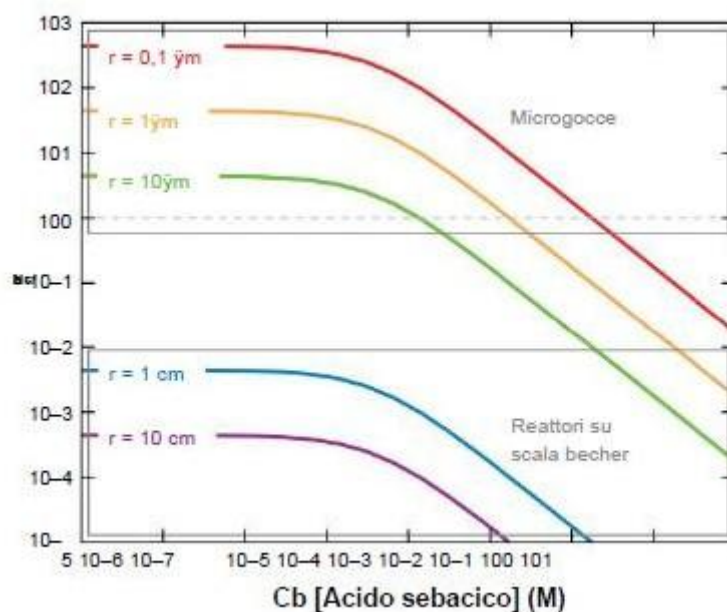


Figure 6. The highlighted areas show the differences between microdroplets and beaker-scale reactors, represented by droplets with radius (r) of 1 and 10 cm.

4. Adsorption Length

The adsorption length (L_{ads}) is a key metric in surfactant literature, representing the distance an interface draws solute from the bulk to maintain equilibrium. It can be expressed in relation to the surface fraction ($N_{s/b}$), highlighting the link between (L_{ads}), droplet size (r), and surface fraction. For small concentrations of sebacic acid, (L_{ads}) can reach micrometer scales, while it decreases to nanometers at higher concentrations.

This reflects the nonlinear relationship between surface and bulk concentrations in the Langmuir equation. Comparing microdroplets to larger containers, (L_{ads}) / r is significantly higher for microdroplets, indicating that solute concentrations are lower relative to macroscale systems. When (L_{ads}) > r , the microdroplet's surface and bulk concentrations differ substantially, impacting reaction kinetics.

A study by Bzdek et al. demonstrates the differences in surface tension between microdroplets and flat surfaces at varying surfactant concentrations [9].

For example, at a Triton X-100 concentration of 0.2 mM, the surface tension of microdroplets is much higher than that of flat surfaces, indicating altered solute partitioning behaviour.

Using the Szyszkowski equation, the Langmuir equilibrium constant can be determined, showing that the surface coverage in microdroplets is significantly affected by the interface. The concentration of adsorbed surfactant at equilibrium is calculated using equations that account for the surface-to-volume ratio of the droplet. The results indicate that smaller droplets require much higher surfactant concentrations to reach similar surface tensions as larger systems.

Ultimately, this analysis reveals that microdroplet surfaces exhibit distinct behaviours compared to their macroscale counterparts, demonstrating the influence of droplet size and surfactant concentration on surface properties.

5. Reacto-Diffusive Length

The reacto-diffusive length (L_{rxn}) is an important characteristic in microdroplet chemistry, representing the average distance a molecule travels before reacting.

It is defined as $L_{rxn} = \sqrt{D/(C_{AB}k_b)}$, where D is the diffusion coefficient and C_{AB} is the reactant concentration. When reactions are slow relative to diffusion ($L_{rxn} > r$), reactants can interact with the droplet's surface multiple times before reacting, leading to kinetics influenced by both the bulk and interface.

In contrast, if reactions are fast and $L_{rxn} < r$, reactants are likely consumed before reaching the surface, reducing its significance.

In multiphase reactions, particularly those involving gas-phase reactants entering the droplet, L_{rxn} is critical for determining where reactions occur and for establishing concentration gradients when $L_{rxn} \approx r$.

These dynamics are discussed further by Limmer et al. in the same volume.

6. Critical Radius for Mass Transport

A critical radius (r_{crit}) characterizes the mass transfer mode to a curved interface, like a bubble or droplet, which can be governed by either diffusion or kinetic control.

The average time for a molecule in a bulk droplet to reach the surface via diffusion is relatively fast compared to beakers, for example, approximately 2 ms for a droplet with a radius of 2 mm.

In the Langmuir model, the characteristic time for a molecule's kinetic transfer from bulk to surface depends on the surface activity of the solute. For sebacic acid, this time is around 3 ms, significantly quicker than the diffusion time.

The critical radius is where the timescales for diffusion and kinetic transfer are equal.

For sebacic acid, r_{crit} is 0.2 mm. In droplets larger than this radius, mass transfer is limited by diffusion, while in smaller droplets, kinetics dominates.

Understanding these timescales is crucial for interpreting acceleration mechanisms in various reactions, especially in electrospray ionization (ESI) droplets, which may have rapid reaction times compared to mass transfer times.

Additionally, in polydisperse droplet distributions, significant differences in partitioning timescales can occur between submicrometer and supermicrometer droplets.

This discussion primarily applies to stable droplets of fixed size, whereas rapidly evaporating or fissioning droplets may require a more complex analysis due to their dynamic interfaces.

1.2 Redox species formed in Aqueous Microdroplets: Properties and Application

The properties of water are peculiar, unique, and fundamental to life thus making it the most essential resource on the planet.

Water is considered an inert environment for many chemical systems.

When microscopic-sized aqueous microdroplets are generated, they exhibit unique properties. These microdroplets can accelerate or trigger redox chemical reactions compared to the same reactions in a solution that does not use external catalysts or oxidants.

The monomeric radical cation of water in the gas phase, $\text{H}_2\text{O}^{+\bullet}$, is easily formed by direct ionization of water vapour. Smyth observed in 1923 in a mass spectrum an ionic species at m/z 18 ion attributing it to $\text{H}_2\text{O}^{+\bullet}$ radical cation.

The primary redox species generated by ESI of water due to the electric field at or near the microdroplet/air interface, is the water radical cation-radical anion pair, $\text{H}_2\text{O}^{+\bullet}\text{---}\text{H}_2\text{O}^{\bullet-}$.

It has been hypothesized that this species is formed by the high electric field of about 10^9 V/m at the air-water interface in microdroplets due to the formation of electric double layers, the alignment of free O-H bonds of interfacial water molecules, or the partial charge transfer resulting in the interfacial $\text{H}_2\text{O}^{+\bullet}\text{---}\text{H}_2\text{O}^{\bullet-}$ pairs.

Recently, Chen et al. have developed a corona discharge-based device for the production of water radical cations in air by introducing a moist inert gas into the discharge volume. Direct ionization of water was achieved in air and this allowed detection of water cations by mass spectrometry.

Intact $\text{H}_2\text{O}^+\bullet$ ions were accompanied by a dominant mono-solvated ion, $\text{H}_2\text{O}^+\bullet(\text{H}_2\text{O})$ and by higher solvated form.

The relatively high abundance of the water dimer radical cation hints at its exceptional stability compared to the polysolvated species. The water radical cation is considered the strongest oxidant in aqueous solutions, with a standard $\text{H}_2\text{O}^+\bullet/\text{H}_2\text{O}$ redox potential greater than 3V.

Its use has limitations such as the high energy required for its generation and the rapid reaction of the water radical cation in the presence of proton acceptors, such as water itself.

A work by Ben-Amotz proposes that the charged water pair $\text{H}_2\text{O}^+ \text{H}_2\text{O}^-$ exists only at a concentration of 3M, when there is initial delocalization of electrons along the hydrogen bond between two adjacent water molecules, with subsequent electron transfer.

A calculation suggests that this process can be greatly enhanced at the interface where an asymmetric hydrogen bond is broken, facilitating charge delocalization. Aqueous microdroplets having large interfacial planes are perfect for facilitating the generation of water radical cations.

Microdroplets possess unique characteristics, including a strong electric field, an acidic environment, partial solvation of reactants, low water content, and a high local concentration of redox species. These factors favour the generation of water radical cations and promote the desired oxidation reactions, reducing the unwanted decay of these reactive species.

The formation of the electric field is based on the alignment of water molecules, with pendant O-H bonds oriented perpendicular to the surface, and possible electron transfer leading to water radical pair [10].

The strong interfacial field is considered the driving force after spontaneous oxidation and reduction in microdroplets.

The electric field can facilitate the separation of charged water molecules, resulting in the formation of cationic and anionic radical pairs.

In water-poor environments, such as at the droplet surface or in organic solvent droplets containing trace amounts of water, the generated cationic

water radical can directly oxidize the substrate producing the oxidized product.

In contrast, under water-rich conditions, such as in the core of water droplets, the cationic water radical would have a short lifetime and react rapidly with water, releasing hydroxyl radicals and hydrogen peroxide.

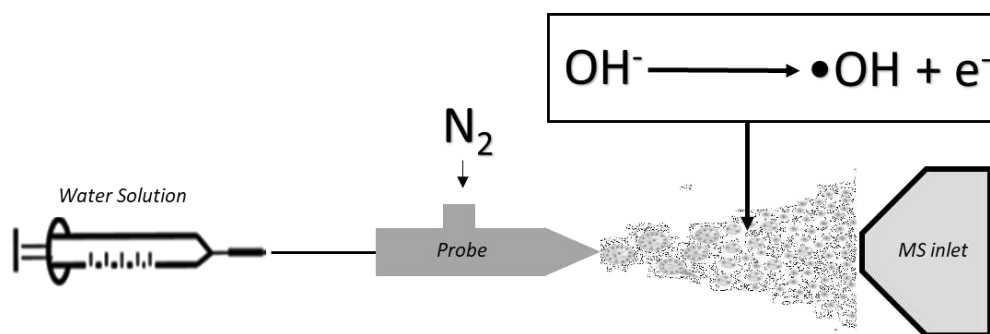


Figure 7. Image of a standard mass spectrometry setup used for studying microdroplet chemistry and electron-mediated redox processes.

Moreover, the high ESI electric field allows the extraction of electrons from hydroxide ions (OH^-) in water producing hydroxyl radicals ($\bullet OH$), Figure 7.

Electrochemical experiments define that the oxidation potential of OH^- to $\bullet OH$ is at least 0.7 V below the tabulated values at the surface of air bubbles in water, so the electron-producing reaction of OH^- should be easier to achieve at the air-water interface than in solution [11].

The $\bullet OH$ radicals generated in microdroplets are not charged and cannot be analyzed by the mass spectrometer, nevertheless, the $H_4O_2^+$ cation was observed in water ESI condition and was structurally characterized as the combination of $\bullet OH$ with H_3O^+ via hydrogen bonding.

Also, the formation of the hydrogen peroxide H_2O_2 by combination of two $\bullet OH$ radicals leads to the formation of strong oxidizing agents in water microdroplets.

1.3 Reaction Acceleration in Thin Film generated by Microdroplets Deposition

Thin films share common chemical properties with microdroplets.

The characteristics shared by both thin films and microdroplets are the high surface-to-volume ratios and the degree of confinement of the reagents.

The difference is that thin films can have interfaces with multiple phases, such as immiscible hydrophobic and hydrophilic solvents and solids precipitated after solvent evaporation, while microdroplets are homogeneous systems.

When the solvent evaporates, new phases form, generating highly complex confined reaction systems.

Moreover, the lifetimes of microdroplets restrict reaction times and limit yields for reactions that are not very highly accelerated.

The need to evaporate large volumes of solvent represents an additional concern in designing scaled-up experiments.

Thin films of reagents might be a better choice for further scale up given their longer reaction times compared to those in microdroplets although the lower surface area has the opposite effect.

When the deposition rate and solution evaporation rate are balanced, the thin film is expected to reach its maximum thickness and to move upwards at a constant thickness as the reagent spray continues to be added and reaction products are precipitated.

A significant and unusual acceleration of the reaction is observed in thin films generated using droplets produced by sonic spray, even when uncharged.

Given these established capabilities, a significant question is whether accelerated reactions can be scaled up to allow meaningful use in chemical synthesis.

For example, in a study by Cooks et al [12], the researchers took advantage of the limited volume of a thin film to study the Claisen-Schmidt base catalyzed condensation of 6-hydroxy-1-indanone with various substituted benzaldehydes, focusing on the acceleration of the reaction rate as a function of mass.

They established optimum conditions for continuous product deposition by simultaneous addition of the reaction mixture and evaporation of the solvent.

They achieved yields of 74% and production rates of 98 mg h⁻¹ in a very simple experimental system that could be scaled up for greater volumes.

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2. Experimental Methods

2.1 ESI Z-Spray

The studies about the reactions within confined volume were performed by using the ESI Z-spray source of a Waters Q-ToF Ultima mass spectrometer.

Ambient pressure ionisation sources are characterised by the fact that the desorption and ionisation of the analyte takes place at ambient pressure.

The advantage of using an ambient pressure source over a reduced pressure source is the ionisation efficiency, which is increased by a factor of one to ten thousand, and the ease of use.

The electrospray ionisation (ESI) process can be divided into three steps:

- I. Nebulisation; the analyte is contained within charged microdroplets.
- II. Desolvation; increase in analyte concentration due to evaporation of solvent.
- III. Formation of gaseous ions.

Nebulisation occurs when the solution containing the sample passes through a capillary to which a voltage is applied.

The electric field allows electrical charges to form on the surface of the liquid exiting the capillary, overcoming the surface voltage and forming the charged microdroplets.

The potential between the needle from which the spray comes out and the focuser is one of the parameters that is often varied to favour ionisation.

The resulting spray is subjected to a coaxial flow of heated N₂ gas, which has the function of preventing spray dispersion and inducing desolvation, which is also favoured by the heating of the capillary. The desolvation temperature can be varied from 25 to 350 °C. In our apparatus, the actual temperature of the and of the thin film formed by their deposition can be measured using a thermocouple as reported in Figure 1.



Figure 1. The actual temperature of the microdroplets/thin film is determined intercepting the liquid beam with a thermocouple placed on the stainless-steel target surface.

The Z-spray source manufactured by Waters for its mass spectrometers is an efficient adaptation of the standard inline spray source. The source is defined Z by the shape of the ion trajectory before it enters the mass spectrometer analyser.

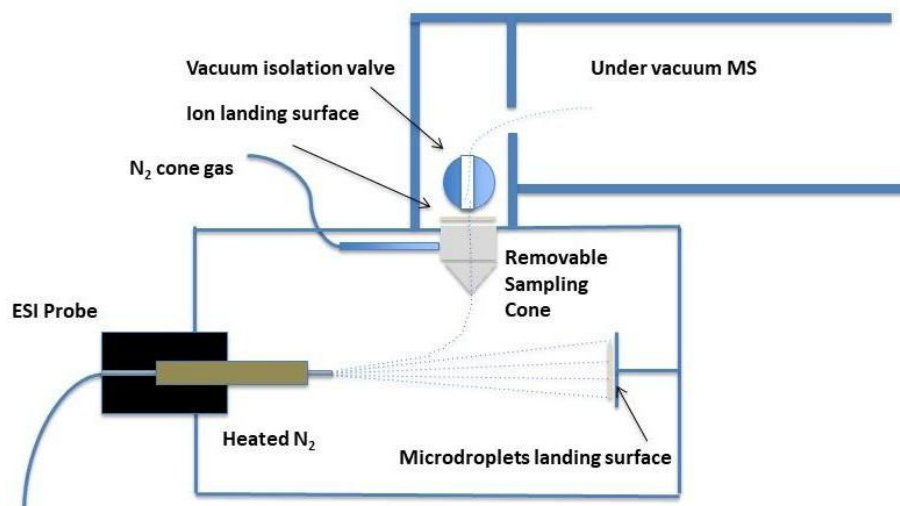


Figure 2. Z-ESI schematic representation.

The electrical potentials of the source cause the ion beam to be deflected towards the orifice and then further deflected to pass through the extraction cone. After the spray is formed, the charged droplets tend to continue in the electric field according to their initial momentum. Neutral solvent molecules are not affected by the electric field.

Desolvation is assisted by the flow of gaseous N₂. Removal of residual solvent and neutrals continues after the skimmer.

With conventional sprays, the ions enter the skimmer along linear trajectories. This characteristic results in the neutral counterpart also reaching the skimmer and depositing on the metal walls of the skimmer, which can cause long-term clogging that reduces the sensitivity of the instrument.

The Z-spray source avoids this problem by separating the trajectories of neutrals and ions, as only the latter are affected by the electric fields. This is achieved by positioning the skimmer at right angles to the droplet trajectory.

The Z-spray path increases sensitivity and instrumental performance, and also provides better access to the microdroplet formation environment, allowing easy manipulation of the system. In addition, the distance between the capillary and the instrument inlet as well as the distance between the capillary and the target plate can be varied.

With the ESI Z-spray modified source it is possible to collect the microdroplets that survive to the coulombic explosion onto a target stainless steel surface creating a stable thin film whose confined volume retains the peculiar microdroplets reactivity. The analysis of the reaction product formed in the thin film can be accomplished with the same mass spectrometer apparatus used to produce the microdroplets.

The reaction progress is qualitatively evaluated by measuring the conversion ratio (CR) using the general formula $[P1]/([R] + [I] + [P1] + [P2])$, where P1 represents the ionic intensity of the product, while [R], [I], and [P2] correspond to the ionic intensities of the reagent, reaction intermediates, and by-products, respectively.

Apparent acceleration factors (AAF) of the reactions are also calculated by comparing the intensity ratio between products and reagents in the

droplet deposition process with the corresponding value measured for the bulk reaction, according to the equation $([P]/[R])_{\text{droplets}}/([P]/[R])_{\text{bulk}}$.

2.2 Scanning Electron Microscope

The scanning electron microscope (SEM) is a type of electron microscope that reproduces the surface of a sample by scanning it with a high-energy electron beam, Figure 3. The electrons interact with the atoms that make up the sample, producing signals that contain information about the topography of the sample surface, its composition and other properties such as electrical conductivity.

The different types of signals produced by the SEM include secondary electrons, backscattered electrons, characteristic X-rays, sample current and transmitted electrons.

Secondary electron detectors are common to all SEMs, but it is rare for a single machine to have detectors for all possible signals. The SEM can therefore produce very high resolution images of a sample surface, revealing details smaller than 1-5 nm.

Because of the very narrow electron beam, SEM micrographs have a large depth of field and produce a characteristic three-dimensional appearance that is useful in understanding the surface structure of a sample.

A wide range of magnifications is possible, from about 10 times (equivalent to a powerful hand lens) to over 500,000 times, which is about 250 times more than the magnification capacity of the best optical microscopes.

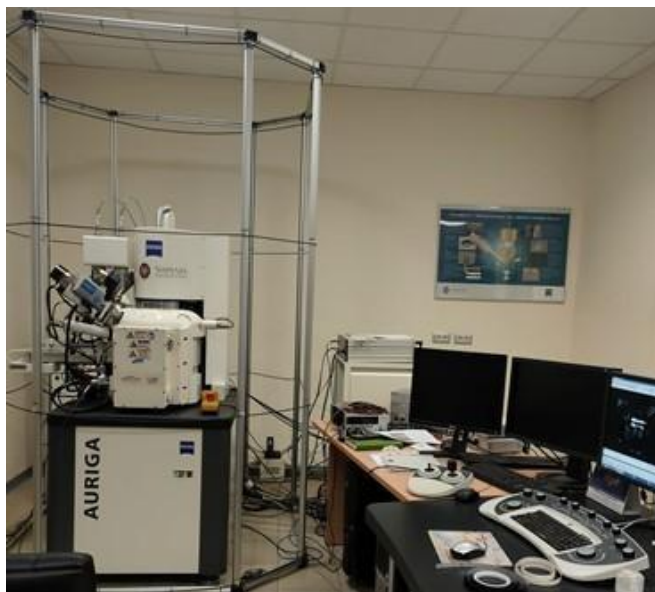


Figura 3. *Photo by HR-FESEM (Field Emission Scanning Electron Microscopy ad alta risoluzione), template AURIGA Zeiss – Laboratory di nanotecnologie e nanoscienze (SNN) – Università La Sapienza (Roma).*

2.3 Cyclic Voltammetry (CV) and Differential Pulse Voltammetry (DPV)

Cyclic voltammetry is a type of potentiodynamic electrochemical measurement. It is one of the most widely used electrochemical methods due to its relative simplicity and high information content.

In a typical cyclic voltammetry experiment, a redox sample is electrolysed (oxidised and reduced) by placing it in contact with the surface of an electrode and then making that surface sufficiently positive or negative in terms of voltage to force the transfer of electrons.

The surface is brought to a certain voltage with respect to an Ag/AgCl reference electrode, the electrode potential is raised to a higher or lower potential at a linear rate, and finally the voltage is returned to its original value at the same linear rate. The electrode potential ramps linearly with time as shown. This ramping is known as the experimental scan rate (V/s). The redox species can gain electrons from the surface or transfer electrons

to the surface when the surface becomes sufficiently negative or positive and reaches the reduction potential of the analyte. This results in a measurable current in the electrode circuit. The potential is measured between the reference electrode and the working electrode and the current is measured between the working electrode and the counter electrode. This data is then plotted as a current against potential. If the redox couple is reversible, then when the applied potential is reversed, a potential is reached which re-oxidises the product formed in the first reduction reaction and produces a reverse current. This oxidation peak will usually have a similar shape to the reduction peak. This provides information about the redox potential and electrochemical rate of the compound. Cyclic voltammetry is a useful technique for studying the mechanism of electron transfer. Electron transfer in biological systems is directly related to conformational changes, surface binding and proton transfer.

To understand the mechanism of an electrolytic reaction, including enzyme catalysis, and to develop biosensors and biocells, electrochemical studies of electroactive molecules bound or adsorbed on electrode surfaces are useful. Figure 4 shows an example of a cyclic voltammogram of a reversible redox species placed on an electrode surface. The term reversible means that the oxidised and reduced species follow a Nernstian equilibrium.

In Figure 4 it can be seen that the cathodic and anodic peaks are symmetrical and the current is maximum at potential E° .

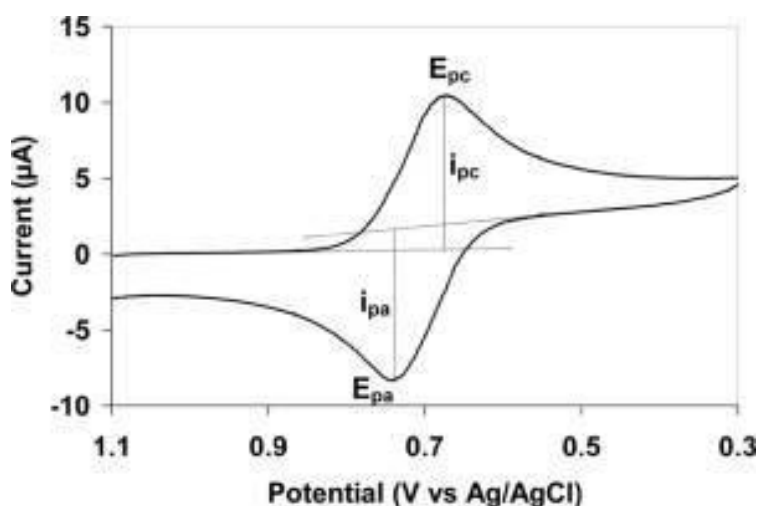


Figure 4. Typical cyclic voltammogram.

In contrast to cyclic voltammetry, in pulsed differential voltammetry, in addition to linear scanning, a periodic series (every $0.5 \div 5$ s) of voltage pulses of constant duration and amplitude (usually $40 \div 60$ ms and with an amplitude (ΔE) varying between 5 and 250 mV) is applied to the electrode, resulting in a significant improvement in performance due to a considerable reduction in capacitive current with a consequent increase in sensitivity.

The method appears to be an alternative to linear scanning, although it gives very similar results.

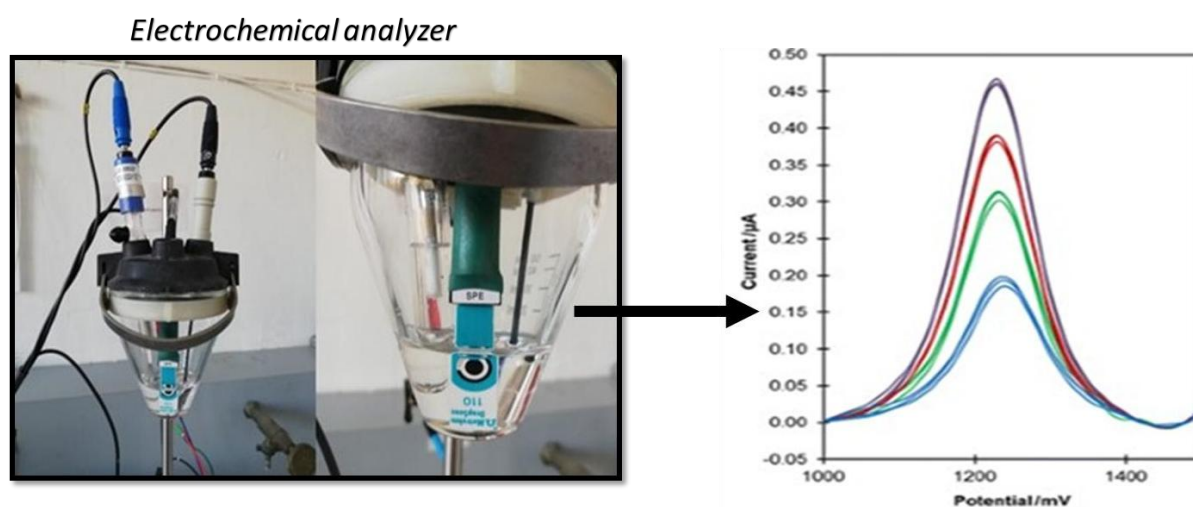


Figure 5. *DPV Voltammogram.*

Since the current is sampled twice, just before the application of the pulse and then in the final moments of the pulse duration, the difference between the second measurement (i_2) and the first (i_1) produces a differential peak voltammogram (Figure 5) in which each signal represents the increase (or decrease) in discharge current for each time interval.

3. Accelerated D-fructose acid-catalyzed reaction in thin films formed by charged microdroplets deposition

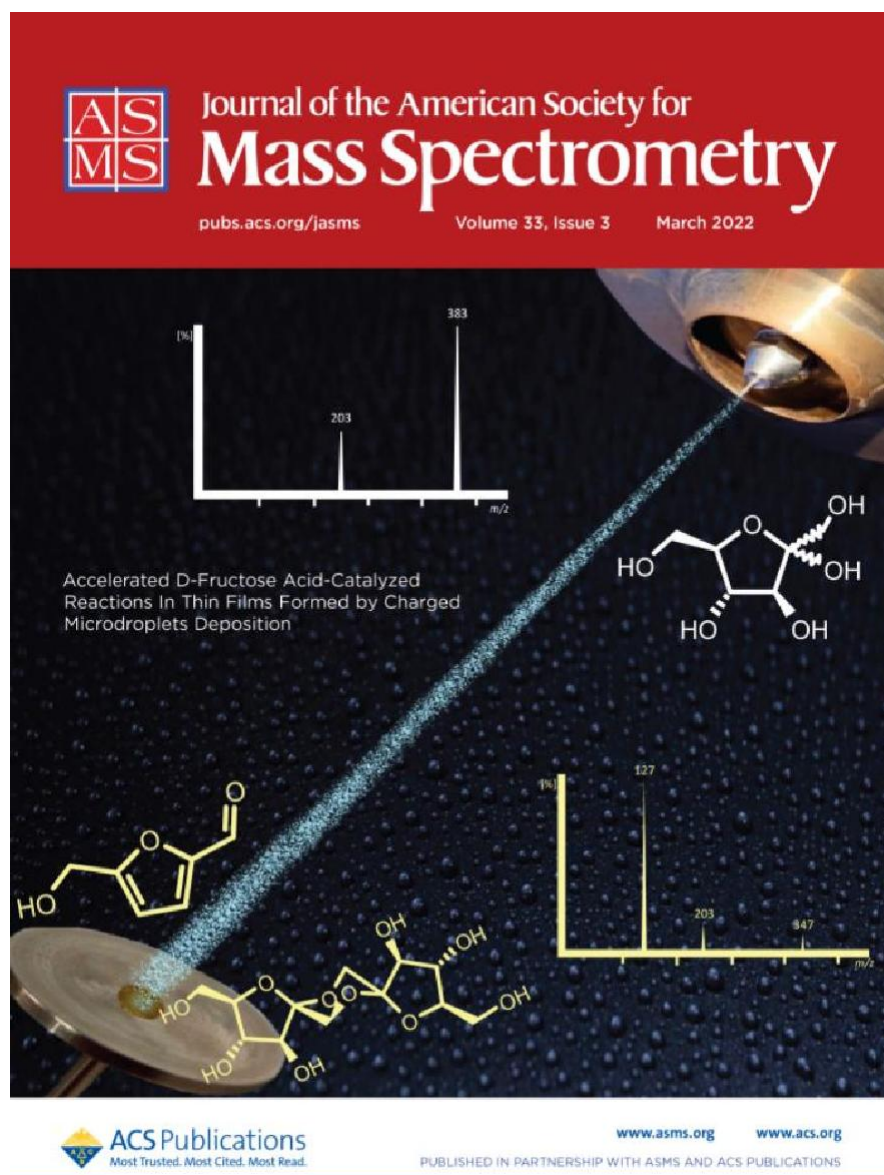


Figure 1. Cover of Journal of the American society for mass spectrometry.

3.1 Introduction

Processes that enable the use of carbohydrates from lignocellulosic biomass as a renewable resource have received much attention over time.

Furan derivatives, such as 5-HMF, are produced by acid-catalyzed dehydration of hexoses, thus providing key building block molecules from 'green' resources [1-2].

Over the last century, the world has become increasingly dependent on oil as the main source of chemicals and energy, with demand rising exponentially and reserves dwindling.

Man has had to look for other sustainable sources of fuel.

Biomass is the most important alternative raw material because it is a carbon-rich source after oil and coal.

Biomass are defined as all materials of biological, animal and plant origin, which can be divided into four categories:

1. forest residues from the wood industry
2. energy crops
3. residues from the agro-food industry;
4. agricultural by-products.

Biomass of agricultural, industrial or natural origin can be converted into chemical substances for energy and industrial use by chemical processes that allow the direct extraction of energy or chemical intermediates that can be used in the energy process.

The main advantage of using biomass compared to fossil fuels is the limitation of the greenhouse effect with lower CO₂ emissions for the same energy and heat production.

Cellulose, as the most abundant natural carbohydrate in nature, is considered a promising raw material for the production of chemicals and fuels, with the added value of being sustainable, biodegradable and biocompatible.

Another purpose for which it may be useful to use this energy source is to solve the problem of waste disposal by eliminating the accumulation of

millions of tonnes of dry matter from agricultural, agro-industrial and forestry by-products and residues.

Biomass is not a renewable resource, but can be considered as such if it is used at a rate below its biological renewal capacity.

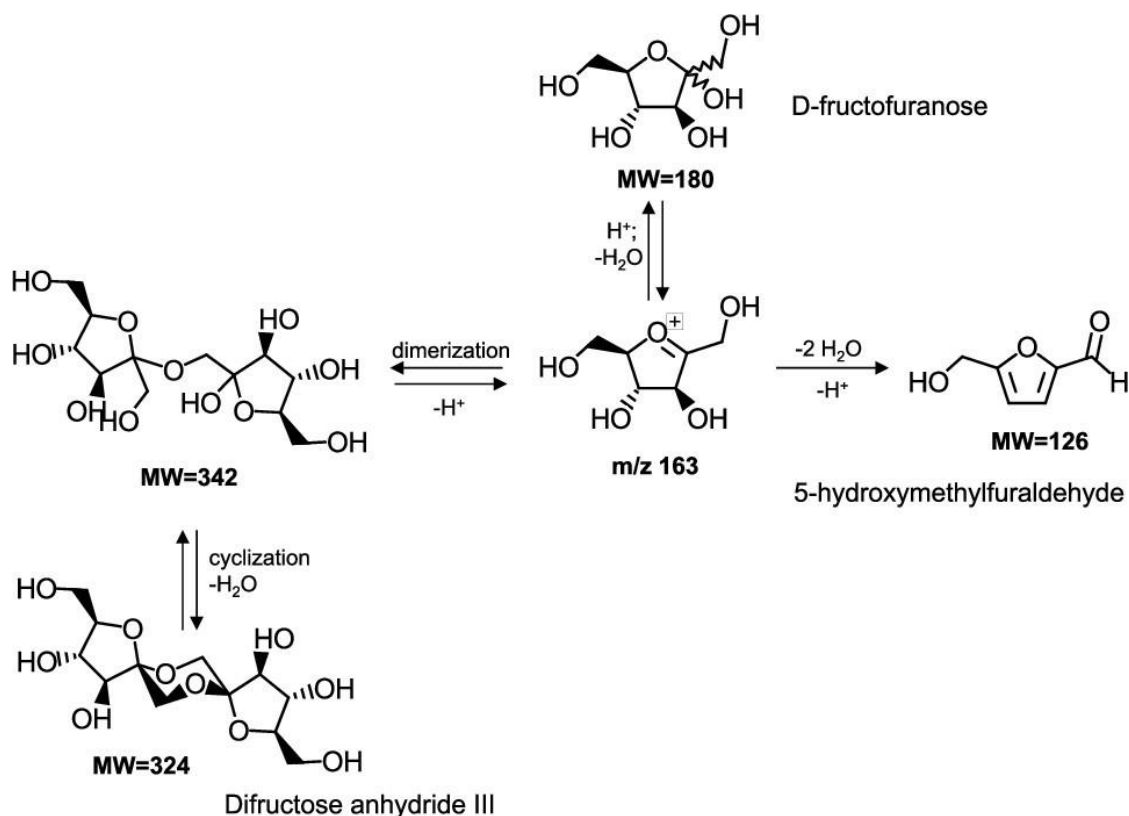
Biomass can be used to produce 'platform chemicals' for the synthesis of commonly used products that are normally synthesised from petroleum. Hemicellulose, cellulose and lignin provide paper, sweeteners, resins, plastics, solvents and chemical compounds.

Another important reaction observed in acid solutions of D-fructose is the condensation of two D-fructose molecules leading to the formation of di-fructose anhydride (DFA) by water loss [3-5].

Polyunsaturated fatty acids are a group of isomeric oligosaccharides that can be used as low-calorie sweeteners [6].

Recently, this class of compounds has been attributed important probiotic properties, such as promoting the growth of beneficial microflora in the gut and protecting the intestinal tract by promoting the assimilation of antioxidants [7].

Dehydration and dimerization are the two different reaction pathways characteristic of D-fructose caramelisation under acidic conditions.



Scheme 1. Mechanistic Pathway of the Acid-Catalyzed Caramelization of D-Fructose in Bulk. The dimerization/cyclization reaction showed involves the same C1-OH groups of two D-fructose molecules leading to difructose anhydride III.

In solution, the fructosiloxocarbenio cation (m/z 163), as seen in Scheme 1, resulting from the initial dehydration of D-fructose, can alternatively dehydrate to form 5-HMF or dimerise and cyclize to form DFA.

In aqueous solutions, the reaction takes place at high temperatures and is catalyzed by strong inorganic acids.

The highest yields of 5-HMF have been obtained from D-fructose, although the bulk reaction has several drawbacks that have limited its industrial application, such as the rapid hydrolysis of 5-HMF to levulinic acid or the formation of insoluble humins.

3.2 Mass Spectrometric Experiments

In this study, hydrochloric acid (HCl) and the potassium bisulfate (KHSO₄) were chosen as metal-free catalysts.

HCl is commonly used in hexose dehydration/dimerization reactions in water, while KHSO₄ has recently been recognised as a selective and safe acid catalyst for the conversion of sugars to 5-HMF [8].

D-Fructose and the catalyst (HCl or KHSO₄) were dissolved in H₂O or CH₃OH/H₂O and CH₃CN/H₂O mixtures to millimolar concentrations, electrosprayed into the ESI source of the instrument, and online analyzed before the microdroplets deposition experiments.

Standard parameters used in the study were: capillary 4 kV, cone 60 V, RF lens-1 70 V, and syringe pump flow 20 $\mu\text{L min}^{-1}$. Nitrogen was used as desolvation gas at a flow rate of 200 L h⁻¹.

The desolvation and source temperatures used to acquire the zero-time mass spectrum in the range 50-500 *m/z*, were 200° and 80 °C, respectively.

Changes in the conversion ratios were in turn evaluated as a function of the source parameters by varying the capillary voltage (0–4 kV), the desolvation temperature (50–350 °C), and the capillary tip-stainless steel target surface distance (0.5–3.0 cm).

The total reaction time of 50 min can be estimated based on a solution volume of 1 mL infused at a rate of 20 $\mu\text{L min}^{-1}$.

The deposition of microdroplets leads to the formation of a solid precipitate, which was rinsed with 1 mL of the same sprayed solvent mixture, and the resulting solution was mass analyzed under the same experimental ESI conditions used to obtain the zero-time mass spectrum.

The amount of the collected solid precipitate was measured by depositing the microdroplets onto a previously weighed portion of an aluminum foil.

Depending on the spray temperature used, the solid recovery in the HCl-containing solutions varied from 90% at low temperatures (the solid mainly contains unreacted D-fructose) to 60–70% at spray temperatures around 150 °C.

The same estimation was critical in the KHSO_4 systems since this salt is hygroscopic and sometimes the amount of the collected mass was higher than the starting one.

For comparison, the same reaction was also conducted in bulk, heating the starting solutions of D-fructose containing HCl or KHSO_4 at 100 °C for 1 h under reflux.

After this time, the reaction mixture was quenched, cooled and analyzed by mass spectrometry.

3.3 Results and Discussion

The stability of the D-fructose acidic solutions at 25 °C was first evaluated by analyzing the starting mixtures.

No sugar decomposition was observed at pH=3 for both the HCl and KHSO_4 systems even after several days from their preparation, whereas at lower pH values an increasing decomposition of the sugar even at 25 °C was observed especially with HCl.

Thus, the D-fructose reactivity was studied by using solutions having pH values ≥ 3 .

The microdroplets generated by the ESI source were collected onto a stainless-steel plate placed in front of the ESI capillary. After the microdroplets deposition, a white or brown solid residue was formed on the metal surface depending on the source parameters used, Figure 2. Subsequently, the solid was recovered and then analyzed.

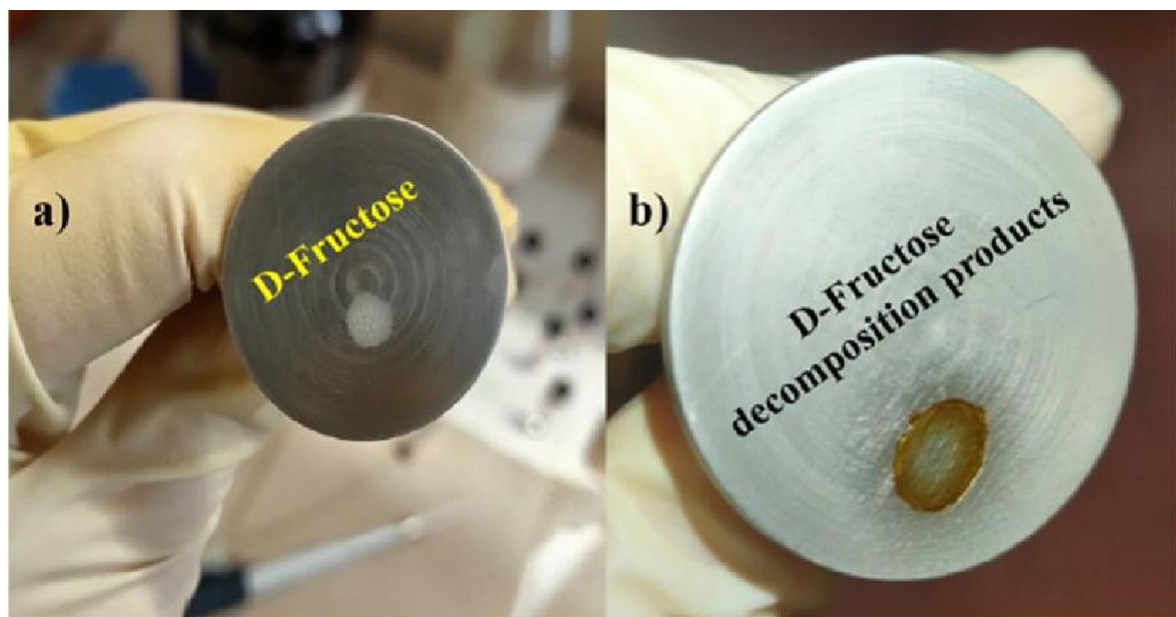


Figure 2. Solid residues recovered after microdroplet deposition: (a) white D-fructose crystals; (b) brown solid containing a mixture of D-fructose decomposition products.

The ESI mass spectra of the $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ 1:1 HCl pH=3 and $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ 1:1 KHSO_4 pH=3 D-fructose solutions, taken as representative of all the investigated systems, are reported in Figure 3 and 4.

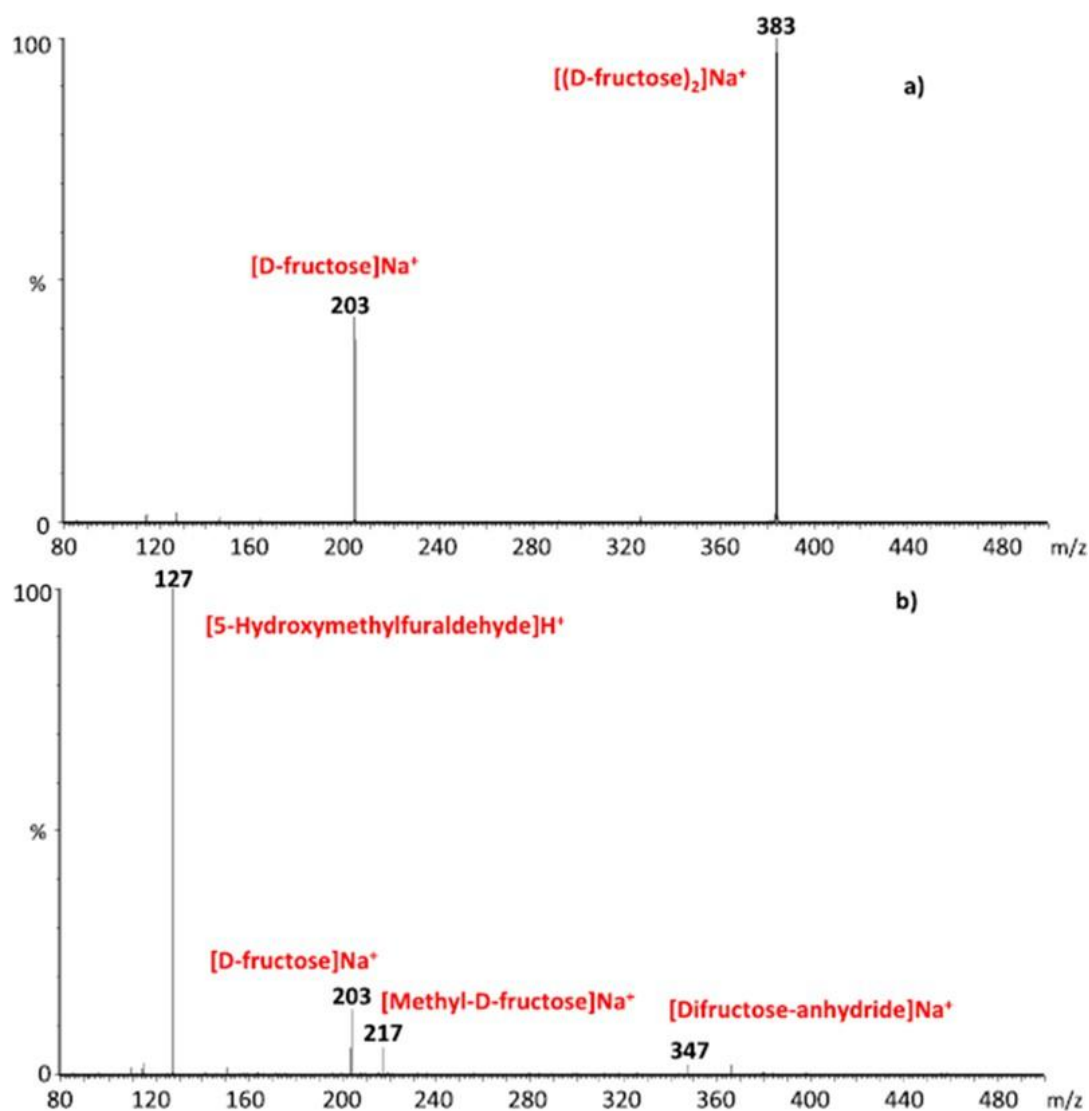


Figure 3. ESI mass spectra of a 10^{-3} M D-Fructose solution at pH=3 using HCl: (a) zero-time ESI mass spectrum of the starting solution; (b) ESI mass spectrum of the solution obtained by rinsing the solid precipitate generated by ESI deposition of 1 mL of the starting solution.

The ESI mass spectrum of the freshly prepared HCl solution, at pH=3 (Figure 3a) is dominated by ions at m/z 383 and 203, corresponding to the $[(D\text{-fructose})_2]Na^+$ and $[D\text{-fructose}]Na^+$ species characteristic of the starting reactive sugar bound to ubiquitous sodium cations.

Comparing this spectrum with that of the reaction products obtained by rinsing the solid residue present on the target stainless steel surface after electrospray deposition of 1 mL of this solution (Figure 3b), is evident a decrease of the intensities of the ions at m/z 383 and 203, reflecting extensive decomposition of the sugar.

The major product generated by the microdroplet deposition reactions is the ion at m/z 127, formally corresponding to protonated 5-HMF.

The dimerization reaction of D-fructose leading to the formation of difructose anhydrides is also catalyzed by HCl, as evidenced by the formation of the [DFAs] Na^+ adduct at m/z 347.

Noteworthy is the formation of the ionic product at m/z 217, corresponding to sodiated methyl-D-fructose, which is indicative of a peculiar microdroplet methylation reaction, presumably due to the presence of methanol as a co-solvent in the ESI sprayed solution.

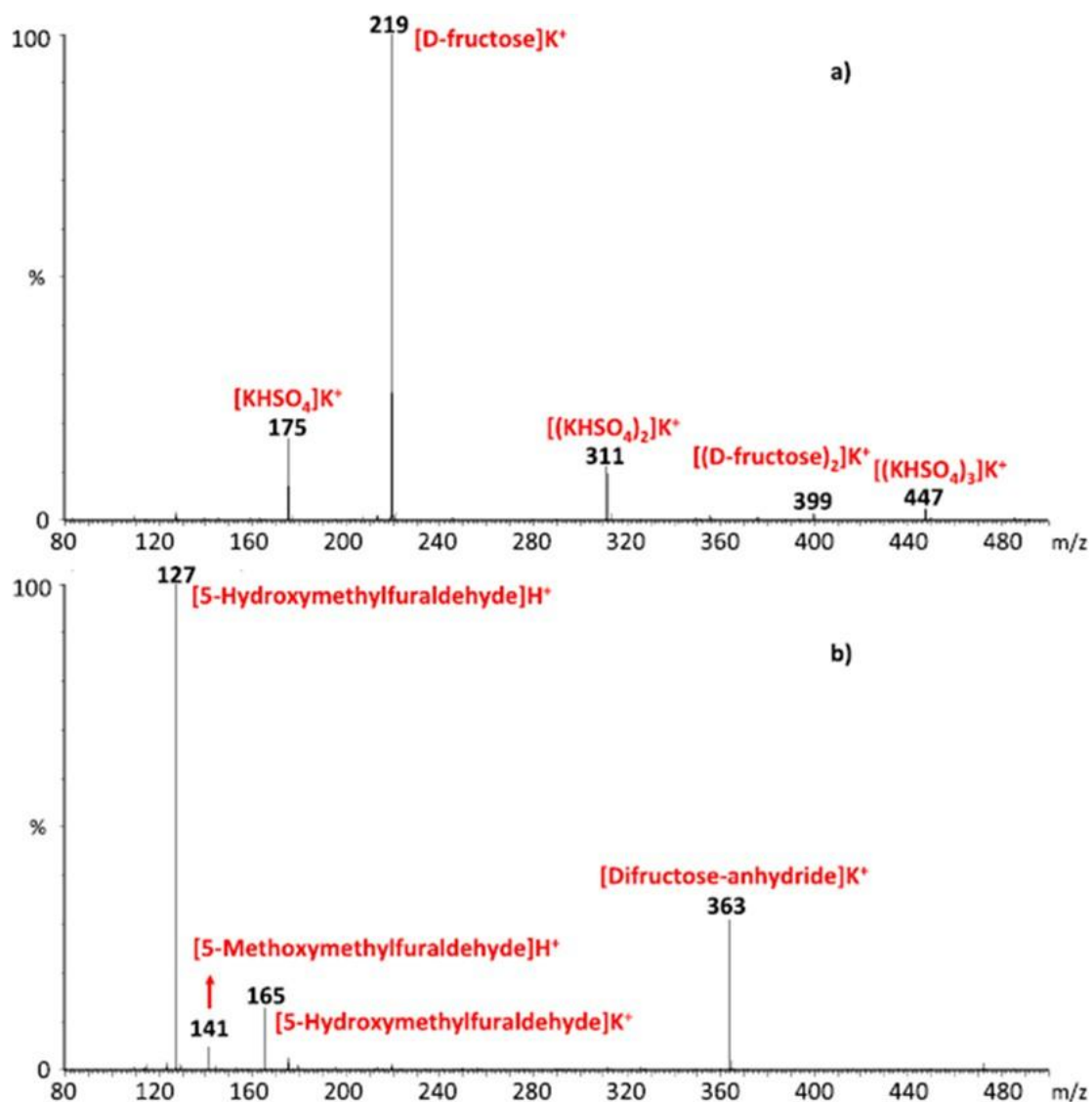


Figure 4. ESI mass spectra of 10^{-3} M D-fructose using KHSO_4 : (a) zero-time ESI mass spectrum of the starting solution; (b) ESI mass spectrum of the solution obtained by rinsing the solid precipitate generated by ESI deposition of 1 mL of the starting solution.

In the KHSO_4 system, the spectrum of the D-fructose/ KHSO_4 starting solution at pH=3 $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (Figure 4a) is dominated by an intense ion at m/z 219 corresponding to the metal adduct between the potassium cation and D-fructose, while the minor ion distribution of the type $[\text{K}(\text{KHSO}_4)_n]^+$ ($n=1-3$) at m/z 175, 311 and 447 can be attributed to the molecular speciation characteristic of this inorganic salts in the gas phase.

As shown in Figure 4b, the ESI mass spectrum of the solid obtained from the microdroplet deposition reaction catalyzed by KHSO_4 is also dominated by the ions $[\text{5-HMF}]\text{H}^+$ at m/z 127 and $[\text{5-HMF}]\text{K}^+$ at m/z 165, which are characteristic of the dehydration reaction of D-fructose.

The products of the methylation and dimerization reactions are also present, as evidenced by the formation of the ion at m/z 141, corresponding to protonated 5-methoxymethylfuraldehyde, and the $[\text{DFAs}]\text{K}^+$ adduct at m/z 363.

The methylation reaction occurs preferentially with the 5-HMF products respect to D-fructose. This is in contrast with the reactivity observed in the HCl-catalyzed reaction.

To test whether the methylation reaction occurs on D-fructose, which subsequently dehydrates to form the ion at m/z 141, or whether the latter species is derived from the direct methylation of 5-HMF, a $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ 1:1 KHSO_4 pH=3 solution of 5-HMF was subjected to the microdroplet deposition reaction under the same experimental conditions used for D-fructose.

Any methylated product was observed, thus demonstrating that D-fructose is methylated prior to undergoing the dehydration reaction leading to the ions at m/z 141. Evidently, KHSO_4 more efficiently catalyzes the dehydration of methylated D-fructose with respect to HCl.

Definitive assignment of the reaction neutral products to 5-hydroxymethylfuraldehyde and 5-methoxymethylfuraldehyde was obtained by comparing the characteristic CID mass spectra of the ions at m/z 127 and m/z 141 with those of protonated standard molecules (Figure 5 and 6).

For both ions, the same characteristic fragmentations occur.

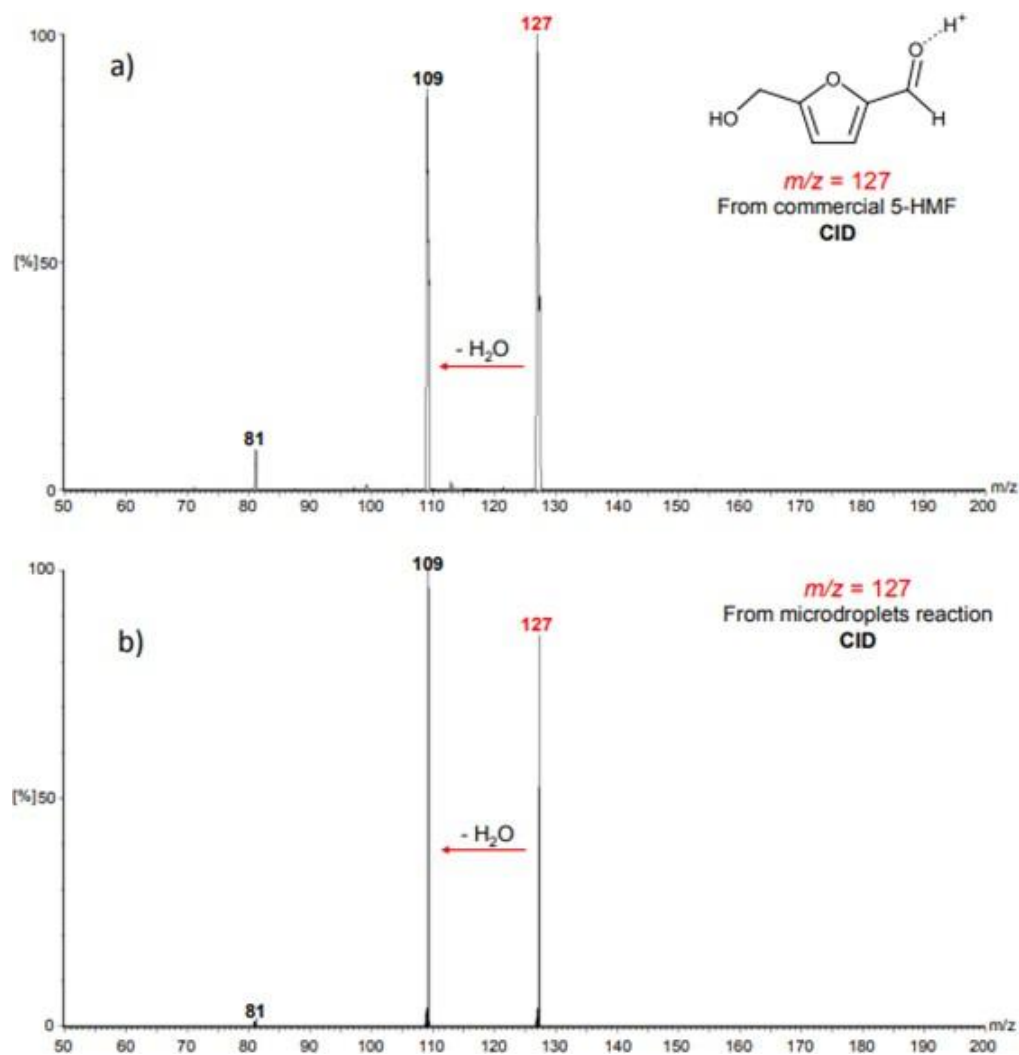


Figure 5. CID spectrum of protonated 5-HMF (a) compared with the CID spectrum of the D-fructose microdroplets dehydration reaction product (b).

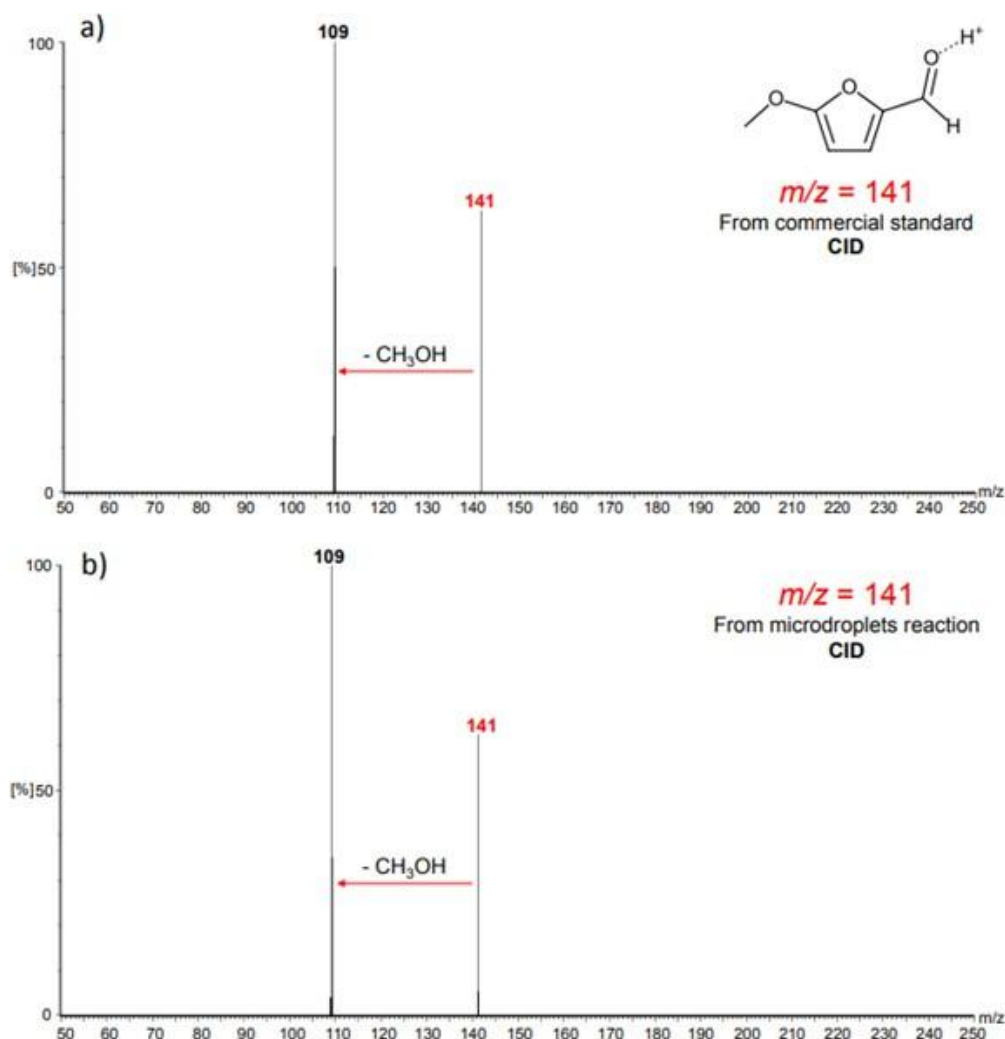


Figure 6. CID spectrum of protonated 5-Methoxymethylfuraldehyde (a) compared with the CID spectrum of the D-fructose microdroplets methylation reaction product (b).

The Na^+ and K^+ cationized DFAs ions at m/z 347 and 363, respectively, do not fragment by collision, suggesting the formation of molecules, such as difructose anhydrides, rather than simple cation adducts between two 162 Da neutral moieties.

The ESI mass spectra of the recovered solutions, obtained by rinsing the solid residue obtained after microdroplet deposition, clearly show that dehydration, methylation and dimerization are the specific D-fructose

reaction pathways common to the two different inorganic acid catalysts used.

To investigate the role of the solution composition on the branching ratios of these reaction channels, different solvent mixtures were tested at different pH values.

Table 1. *D-Fructose Conversion Rates Observed in Various Solvent Mixtures at a Desolvation Temperature of 200°C.*

Catalyst	pH	Solvents	CR _{dehydration}	CR _{dimerization}	CR _{methylation}	Total CR
HCl	3	H ₂ O	8.1			8.1
HCl	3	H ₂ O/CH ₃ CN 1:1	54.8	4.0		58.8
HCl	3	H ₂ O/CH ₃ OH 1:1	60.7	1.7	3.7	66.1
HCl	4	H ₂ O/CH ₃ OH 1:1				
KHSO ₄	3	H ₂ O	51.1	42.2		93.3
KHSO ₄	3	H ₂ O/CH ₃ CN 1:1	80.5	14.9		95.4
KHSO ₄	3	H ₂ O/CH ₃ OH 1:1	72.0	22.3	4.7	99.2

The progress of the reaction was evaluated qualitatively by measuring the conversion ratio (CR) and the conversion ratio of every single reaction channel.

The influence of the solvent composition on the measured conversion ratio was evaluated using three different systems: H₂O, H₂O/CH₃CN, H₂O/CH₃OH.

For the hydrochloric acid catalyst, the pH was also increased to 4.

Among the HCl catalyzed systems, the more acidic 1:1 H₂O/CH₃OH mixture gives the highest conversion ratio of D-fructose to 5-HMF, which strongly dominates over the methylation and dimerisation reactions.

Methylation is absent when the reaction is carried out in H₂O/CH₃CN.

The absence of methylated products in CH₃CN containing mixtures clearly underlines the role of methanol in promoting the methylation

reaction channel. In water the reactions are suppressed. No reactions were observed with HCl at pH values above 3, not even at desolvation gas temperatures above 300 °C.

On the other hand, a total conversion close to 100% was measured for all mixtures studied with KHSO₄. The H₂O/CH₃CN solution at pH=3 is the optimal system for catalysing the dehydration of D-fructose with KHSO₄, whereas the dimerisation reaction increases under conditions that seem to prevent the dehydration channel, such as in the case of a pure water solution.

Comparing all the results, KHSO₄ seems to be the best catalyst for accelerating D-fructose reactions by microdroplet deposition in the slightly acidic condition used, showing a total CR of = 99.2%.

In addition, H₂O/CH₃OH systems at pH=3 containing HCl or KHSO₄ were chosen as model solutions for subsequent experiments.

These systems allow a more extensive decomposition of D-fructose to be observed.

Many studies show that the ESI source parameters are crucial in influencing the conversion ratio of the process [9,10].

In the instrumental setup used, the temperature of the source block does not influence the reactivity of D-fructose, since the microdroplets formed on the way from the ESI needle to the metal plate do not pass through the MS inlet before being collected.

On the contrary, the outcome of the reaction may be influenced mainly by the temperature of the desolvation gas, which promotes solvent evaporation both in the flying microdroplets and in the thin film resulting from their deposition on the solid target.

In the ESI Z-spray source, the nitrogen desolvation gas is heated and delivered in the form of a coaxial sheath to the liquid spray passing through a desolvation nozzle that can be heated up to 350°C.

The temperatures of the desolvation nozzle were varied from 50 to 350 °C and the corresponding effective spray temperatures were measured by a thermocouple intercepting the flow of microdroplets before their deposition on the target, Figure 7.

In general, the measured effective temperatures were about 50 per cent of the set desolvation gas temperature, i.e. a desolvation gas temperature of 200 °C corresponds to an effective spray temperature of about 90 °C.

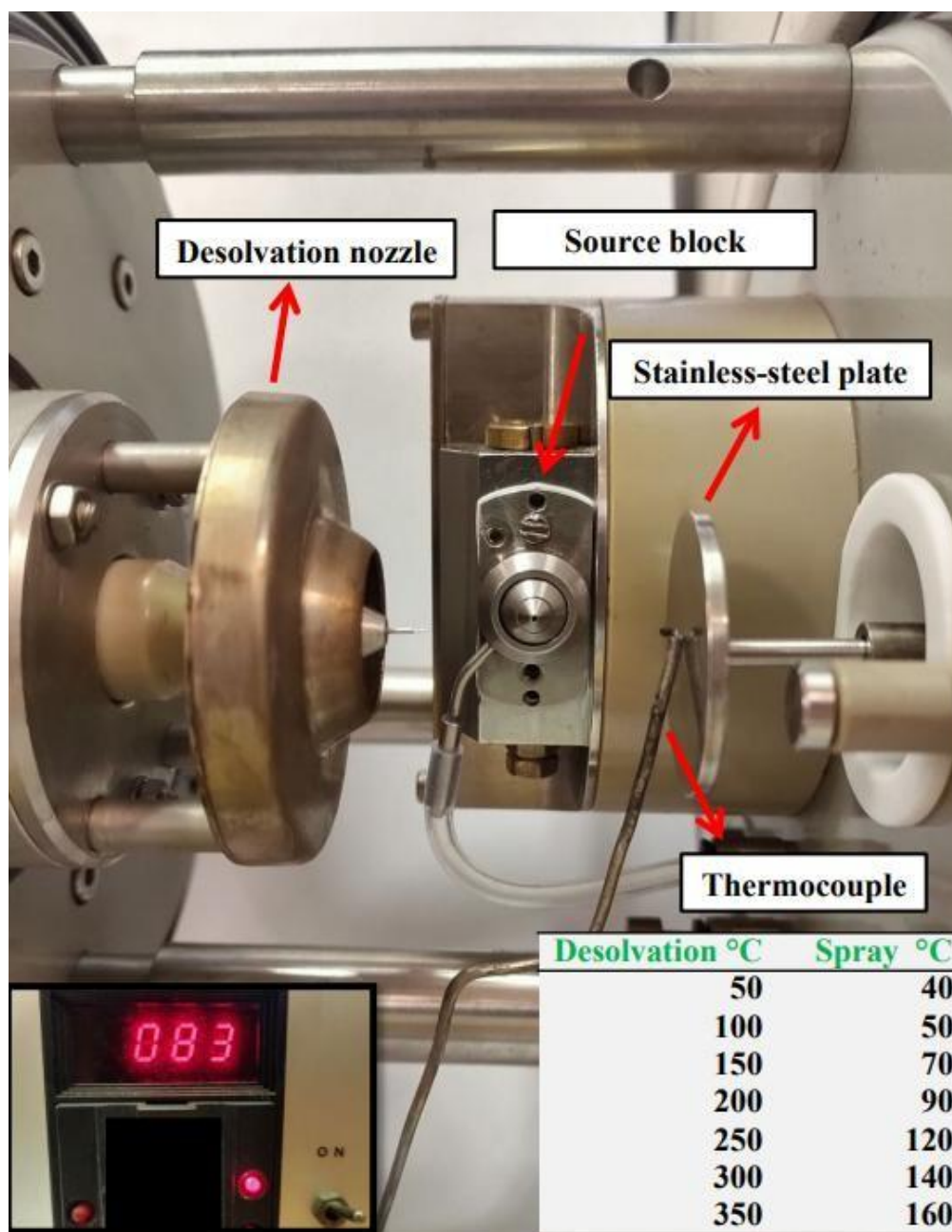


Figure 7. ESI Z-spray source set to spray/thin film temperature measurements. In the table, the desolvation nozzle temperatures and the corresponding spray/thin film measured values are reported. The measured effective temperatures were about 50 % of the set desolvation gas temperature, a desolvation gas temperature of 200 °C corresponds to an effective spray temperature of about 90 °C.

In Figure 8 is reported a picture of the solid separated after the infusion of 1 mL of KHSO_4 D-fructose solutions at different desolvation temperatures. It is evident the deposition of a D-fructose with the solid at low temperature whereas at higher temperatures the brown residue clearly is mainly composed D-fructose degradation products.

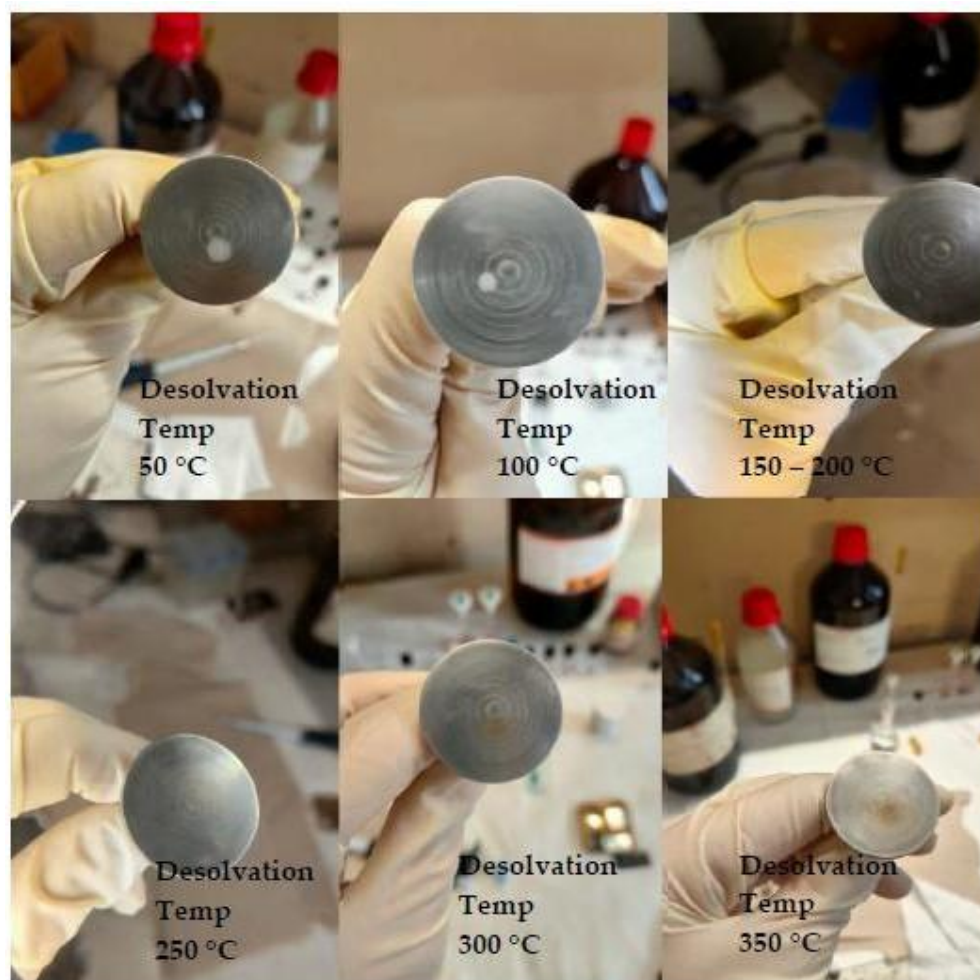


Figure 8. Stains deposited on the stainless steel plate after the infusion of 1 mL of KHSO_4 D-fructose solutions at different desolvation temperatures

The relative intensities of the three HCl and KHSO_4 catalyzed reaction channels are depicted as a function of the actual thin film temperatures in Figures 9 and 10.

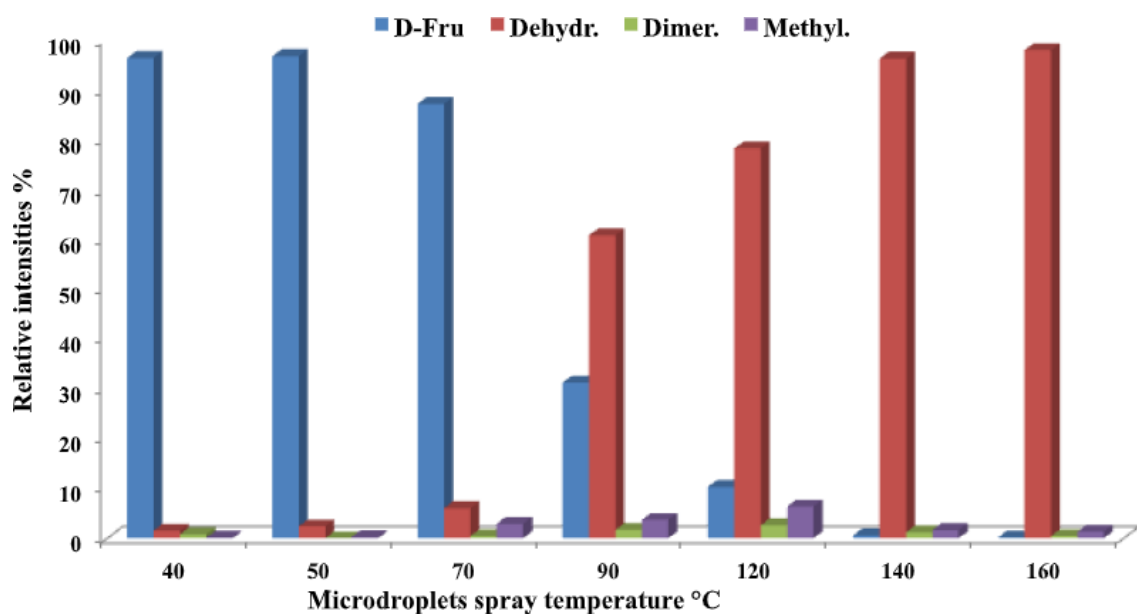


Figure 9. *HCl-catalyzed reaction channel relative intensities (%) versus microdroplet spray temperatures (°C).*

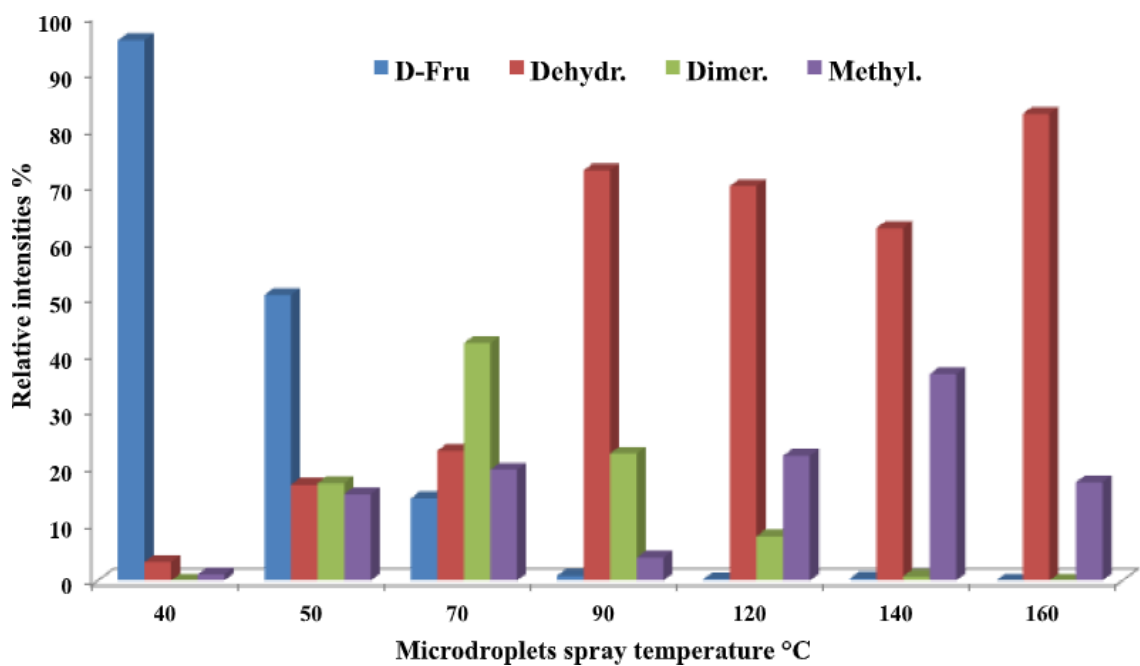


Figure 10. *KHSO₄-catalyzed reaction channel relative intensities (%) versus microdroplets spray temperature (°C).*

Using HCl as a catalyst, the dehydration reaction (Figure 9, red bars) predominates over the other reaction channels at all temperatures tested. The formation of 5-HMF starts at a spray temperature of about 90 °C and reaches a maximum relative intensity of about 100% at 140 °C.

Methylation and dimerization are minor secondary reaction channels, always below 10% of relative intensity, with the methylated product reaching maximum conversion at 120 °C.

As shown in Figure 10, the KHSO₄-catalyzed conversion of D-fructose is already complete at a spray temperature of 90 °C, in contrast to the HCl system where a temperature of at least 140 °C is required for complete decomposition of the sugar.

The yield of 5-HMF in the KHSO₄ system increases progressively with spray temperature until it reaches a threshold of about 90% at 160 °C.

In addition, a temperature of around 90 °C is required to maximise the selectivity of the process towards the dehydration reaction. The optimum temperature to drive the reaction towards DFA production is 70 °C, while the methylation reaction of D-fructose starts at a temperature of about 50 °C and reaches a maximum relative intensity of about 40% at 140 °C.

Comparing the graphs of the two different catalysts used, it is clear that KHSO₄ has a greater ability to promote all the degradation pathways of D-fructose, even at lower temperatures, than HCl.

The special feature of HCl is that it allows the reaction to proceed almost completely towards the selective formation of 5-HMF.

The ESI capillary voltage is another key source parameter that typically influences the nature of the desolvation process leading to microdroplet formation [11,12].

The relative intensities of the three different reaction channels were measured at a capillary voltage of 0 kV and a spray temperature of 50 °C, at which D-fructose is not completely decomposed, to determine whether this parameter influenced the observed reactions. Similar results were obtained without applied capillary voltage compared to experiments with 4 kV capillary voltage, demonstrating that the measured conversions are only slightly affected by the electric field of the ESI source (Figure 11).

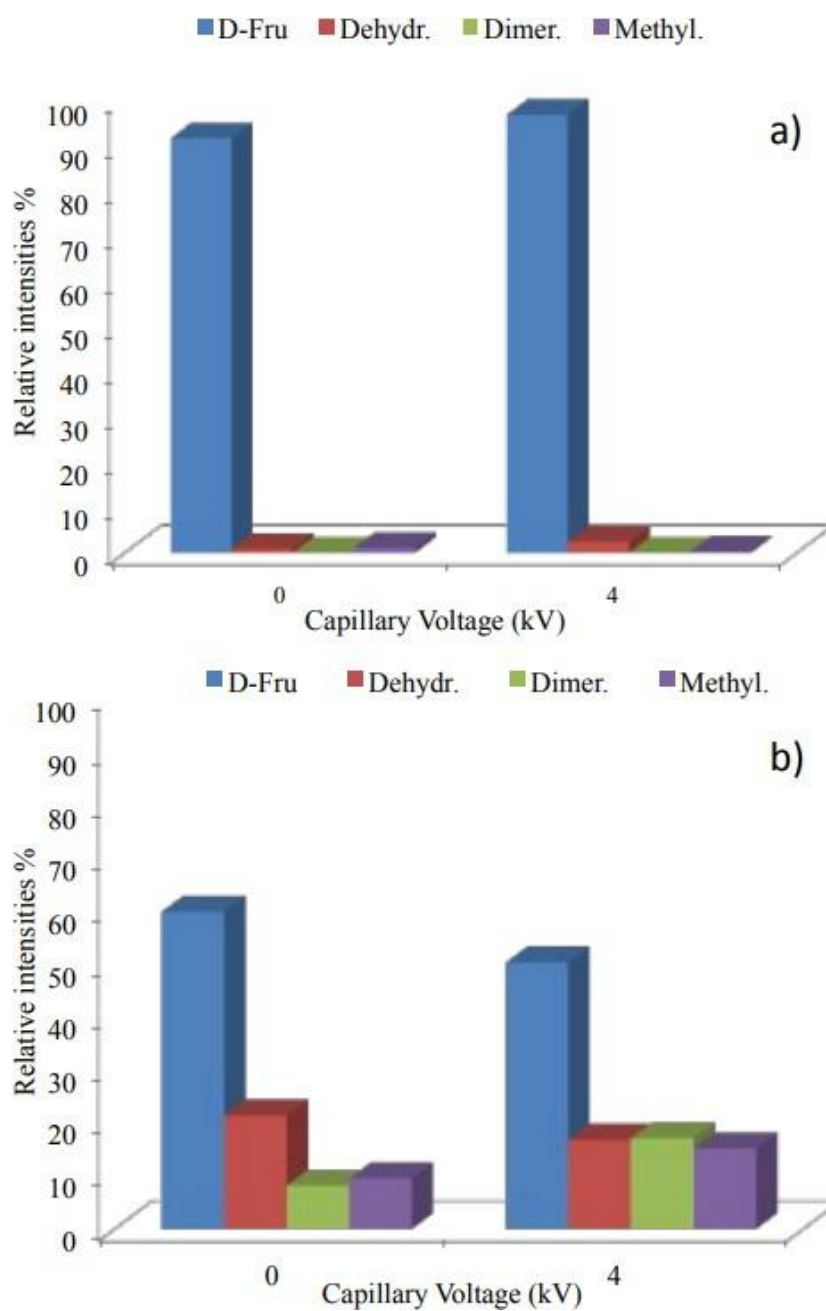


Figure 11. Relative intensities (%) HCl (a) and KHSO₄ (b) catalyzed reaction channels at 0 and 4 kV applied capillary voltage.

3.4 Microdroplets or Thin Films Reactions?

In order to establish the apparent acceleration factors of the reactions observed in the microdroplet thin film deposition process compared to the same reaction in bulk, the same D-fructose $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ 1:1 pH=3 solutions containing HCl or KHSO_4 were heated at 100 °C under reflux for 1 h, under almost the same experimental conditions used to measure the conversion ratios given in Table 1.

The spectra of the HCl solution taken at time zero and after 1 h of reaction are completely superimposable (Figure 12).

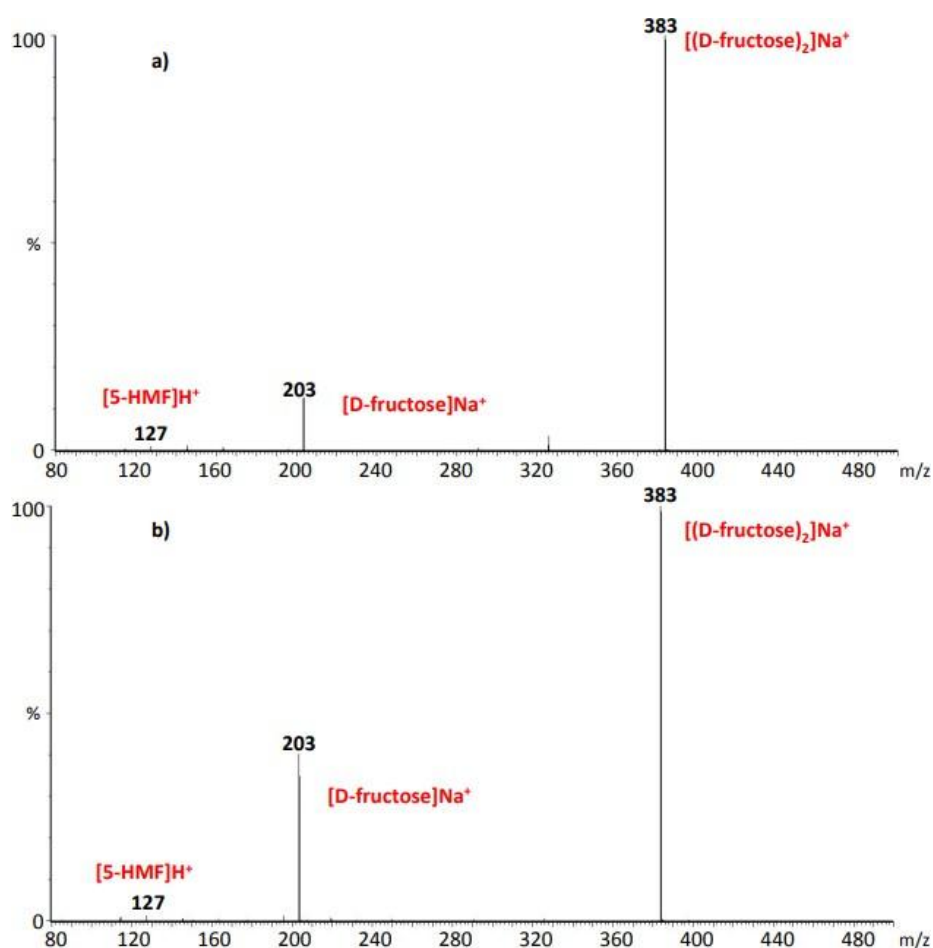


Figure 12. ESI mass spectra of D-fructose $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ 1:1 HCl solution at pH=3 heated at 100 °C under reflux: time zero (a) and after 1 hour of reaction (b).

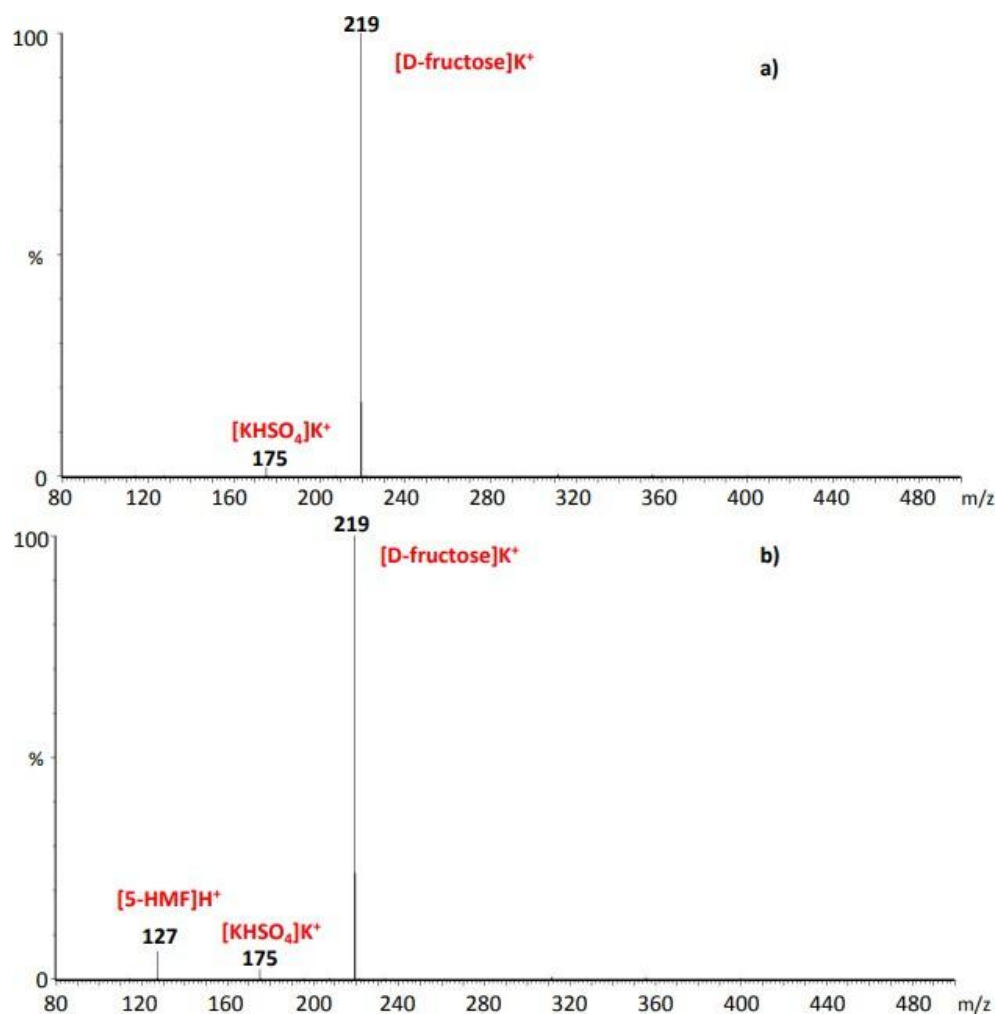


Figure 13. ESI mass spectra of D-fructose $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ 1:1 KHSO_4 solution at pH=3 heated at 100 °C under reflux: time zero (a) and after 1 hour of reaction (b).

Only a very low conversion ratio to 5-HMF was observed for the solution containing KHSO_4 as catalyst (Figure 13).

Thus, the fructose dimerization and methylation reactions catalyzed by HCl and KHSO_4 at pH=3 are characteristic reactions of the microdroplet thin film deposition process and do not occur in solution under the same experimental conditions. KHSO_4 catalyses the dehydration reaction both in the thin film system and in solution at pH=3.

However, considering the dehydration conversion ratio of KHSO_4 of about 7.0% measured in bulk, an apparent acceleration factor of 10.5 can be estimated for the corresponding thin film process.

3.5 Conclusion

D-fructose dehydration, methylation and dimerization products are formed by the rapid desolvation of thin films produced by the deposition of microdroplets generated by electrosprayed D-fructose solutions containing HCl or KHSO_4 as mineral acid catalysts.

The reaction is highly dependent on the catalyst and solvent used, as well as on the desolvation gas temperature.

Interestingly, HCl selectively drives the reaction towards D-fructose dehydration under mild synthetic conditions at which the bulk reaction does not take place. Dilute solution of a 'green' catalyst such as KHSO_4 promotes total sugar conversion into dehydrated, methylated and polymerized products at a spray temperature around 90°C. KHSO_4 -catalyzed D-fructose dimerization and methylation reactions are peculiar of the thin-film system and do not occur in bulk, while KHSO_4 -catalyzed D-fructose dehydration is accelerated by an apparent factor of about 10.5 compared to the same reaction in solution.

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4. Kinetic study of the Maillard Reaction in Thin Film generated by Microdroplets Deposition

4.1 Introduction

The Maillard reaction was first described in 1912 by French chemist L.C. Maillard as a reaction between glucose and glycine that, when heated, resulted in the formation of a brown pigment (melanoidin) [1].

The Maillard reaction (MR) is the primary process responsible for the formation of desirable or undesirable flavored and colored products in cooked foods [2,3]. It occurs in vivo in mammalian organisms where glycosylation is one of the most common protein post-translational modification processes [4-6].

Under certain heating conditions, reducing sugars (such as xylose and glucose) and compounds containing free amino groups (such as amino acids, peptides, and proteins) can react to form a series of "Maillard reaction products" (MRPs).

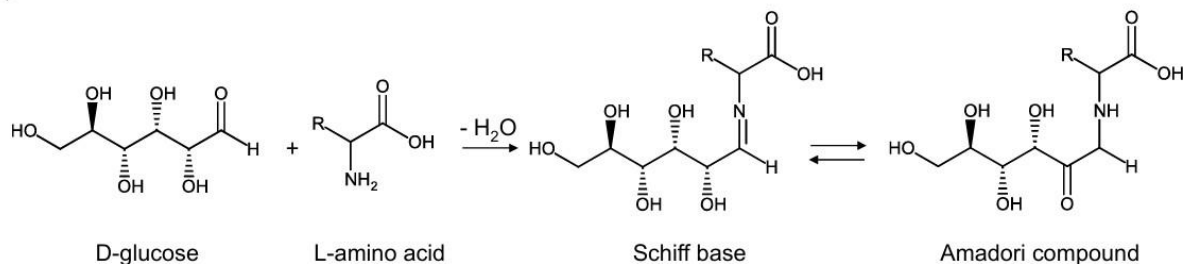
In the initial stage of the reaction, the free amino group of amino acids (especially the L-lysine ϵ -amino groups of proteins, but also the α -amino groups of terminal amino acids) is added to the sugar carbonyl.

This leads to the reversible formation of a Schiff base by removal of a water molecule.

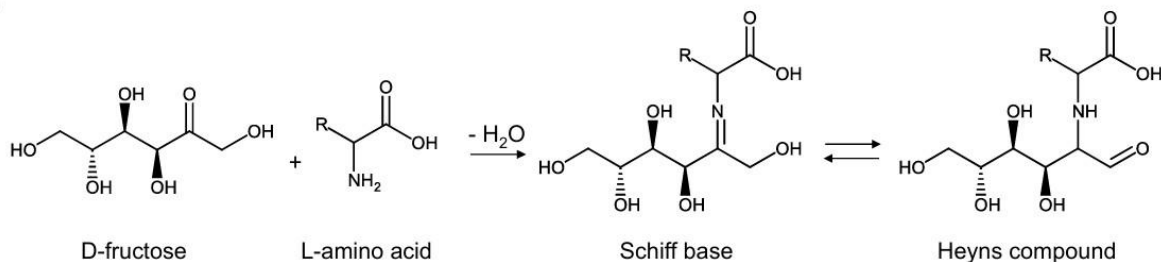
Depending on the nature of the sugar, the formed Schiff base rearranges into Amadori or Heyns products [7-9].

Aldoses such as D-glucose lead to the Amadori compound whereas ketoses such as D-fructose give rise to the Heyns intermediate (scheme1).

a)



b)



Scheme 1. The first step of the Maillard reaction: (a) the reaction involving D-glucose and (b) the reaction involving D-fructose.

Some of the factors that can influence MR are:

- Type of amino acid: The rate of reaction is different for different types of amino compounds; it is higher for amines, lower for amino acids and low for proteins. Different amino acids produce different compounds under the same conditions [10].
- Type of sugar: Reducing sugars are an essential substrate for the Maillard reaction; different types of sugars react at different rates depending on their structure. In general, monosaccharides react faster than disaccharides and pentose sugars react faster than hexose sugars [11].
- The pH value: Different reaction steps are catalyzed by different pH values. It has been observed that in the pH range between 3

and 9, the reaction rate of browning increases with increasing pH [12].

- Temperature and time: The Maillard reaction involves complex reaction steps that have different sensitivities to temperature; it has been observed that as the temperature increases, the number of substrates involved and the speed of the reaction increase, but it has also been seen that an excessive increase in temperature leads to the formation of toxic compounds [13]. It has been deduced that the temperature cannot be too high (less than 180°), but on the other hand, if the temperature is too low, not only does the reaction speed decrease, but the formation of various aromatic compounds is also inhibited [14].
- Water and metal ions: The water content influences the Maillard reaction. In particular, a percentage between 30% and 75% allows an increase in the reaction rate directly proportional to the amount of water [15]. In addition, various metal ions, including cupric and ferric ions, have the ability to promote browning through a catalytic effect [16].

When food is heated, the reaction continues until volatile compounds are formed.

These volatile compounds are usually associated with food flavour and aroma.

From this point of view, the final products of the MR can be used to produce synthetic flavourings that can be added to processed foods [17-19].

However, such volatile compounds are easily lost from foods, so attenuating their volatile degradation products is a promising method for controlled food flavour enhancement.

There are several studies on the preparation of MR intermediates in the aqueous phase using amino acids, but the whole process remains quite complex. Cooks and co-workers studied the accelerated Schiff base formation from the reaction between primary amines and saccharides for saccharide discrimination in tissues and single cells in a thin film reactor

where concentrated solution droplets had been allowed to dry over time [20].

The main objective of the present work was the study of the kinetics of the MR in a thin film containing a fixed amount of reagents whose lifetime was extended over the time by continuous pure solvent microdroplets deposition.

4.2 Results and discussion

To investigate the Maillard reaction in the thin film, the reactivity of aldose and ketose sugars (D-glucose, D-xylose and D-fructose) towards L-glycine, the simplest amino acid, and L-lysine, which carries an additional ϵ -amino group, was studied by collecting the microdroplets generated by the Z-spray ESI source of a Q-TOF mass spectrometer on a stainless-steel surface. The starting solutions were prepared by mixing an equimolar amount of the sugar and the amino acid in a 3:1 H₂O/CH₃OH solution.

The ESI mass spectra of the starting solutions, reflecting the ionic composition of the microdroplets, are shown in Figures 1 and 2.

The spectra of the initial L-glycine/sugar solutions were dominated by the [M]Na⁺ and [M₂]Na⁺ adducts characteristic of the starting sugar bound to the ubiquitous sodium cations. The absence of ionic signals from L-glycine can be attributed to the presence of its zwitterion in solution.

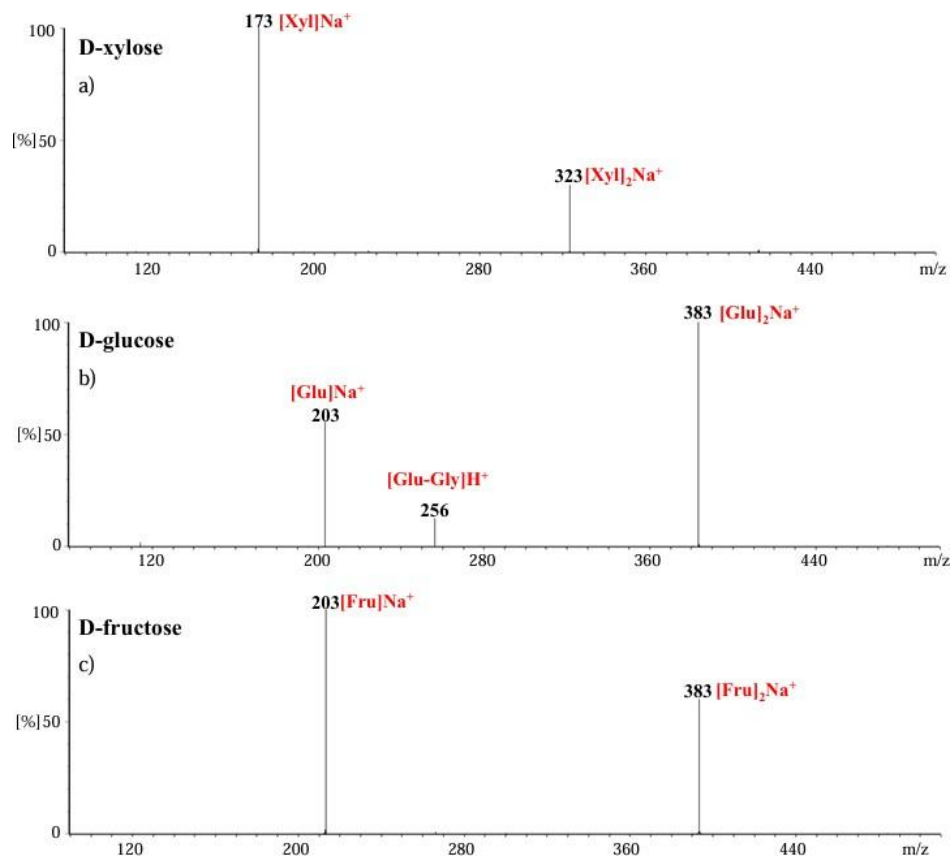


Figure 1. The ESI mass spectra of the starting solutions: a) 1 mL of 1:1 L-glycine/D-xylose 10^{-3} M H₂O/CH₃OH 3:1 solution; b) 1 mL of 1:1 L-glycine/D-glucose 10^{-3} M H₂O/CH₃OH 3:1 solution; c) 1 mL of 1:1 L-glycine/D-fructose 10^{-3} M H₂O/CH₃OH 3:1 solution.

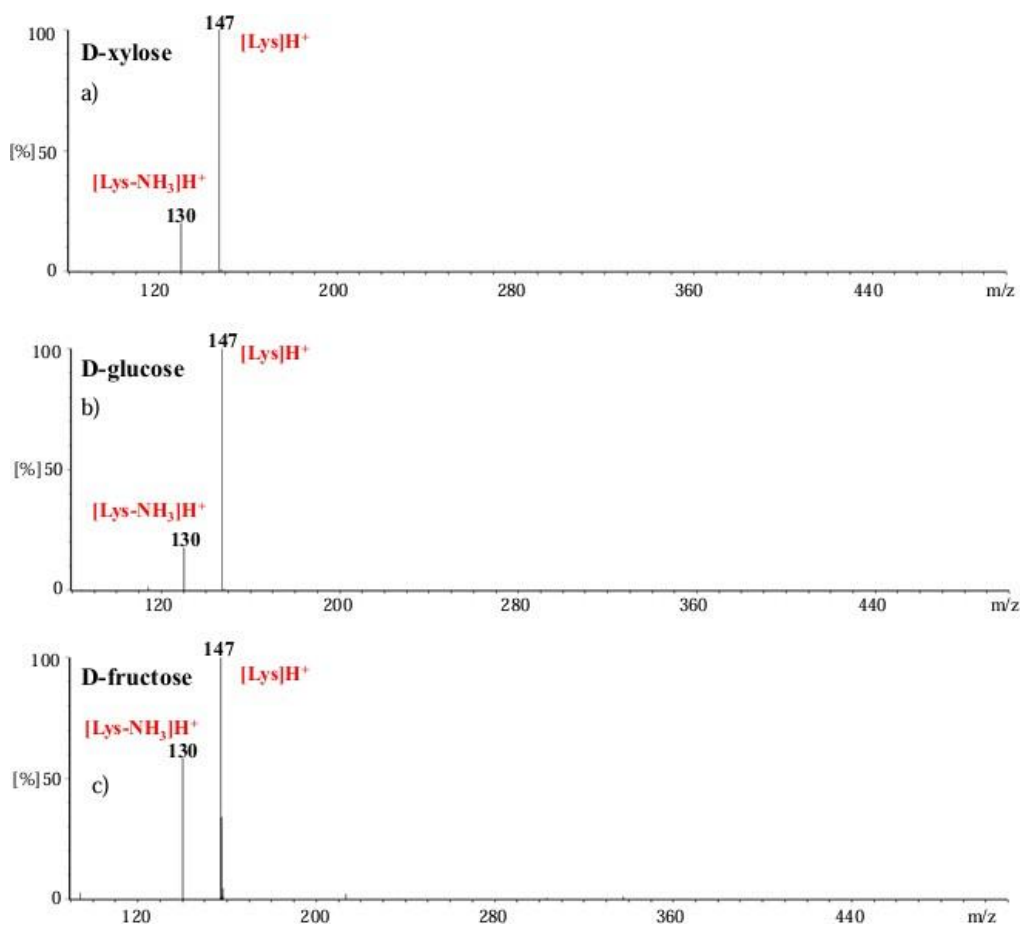


Figure 2. ESI mass spectra of the starting solutions: a) 1 mL of 1:1 L-lysine/D-xylose 10^{-3} M H_2O/CH_3OH 3:1 solution b) 1 mL of 1:1 L-lysine/D-glucose 10^{-3} M H_2O/CH_3OH 3:1 solution, c) 1 mL of 1:1 L-lysine/D-fructose 10^{-3} M H_2O/CH_3OH 3:1 solution.

On the other hand, protonation of the ϵ -amino group of L-lysine under ESI conditions leads to the almost exclusive observation of the $[Lys]H^+$ and $[Lys-NH_3]H^+$ ionic species at m/z 147 and m/z 130, respectively.

No ionic species attributable to MRPs were observed, demonstrating that no reaction takes place in the microdroplet beam prior to its deposition on the solid surface.

During the reaction time, typically 50 minutes, the deposition of the ESI microdroplets on the stainless-steel surface produces a thin film from which a brown precipitate separate.

The spectra of the solids obtained from the thin film system are depicted in Figure 3.

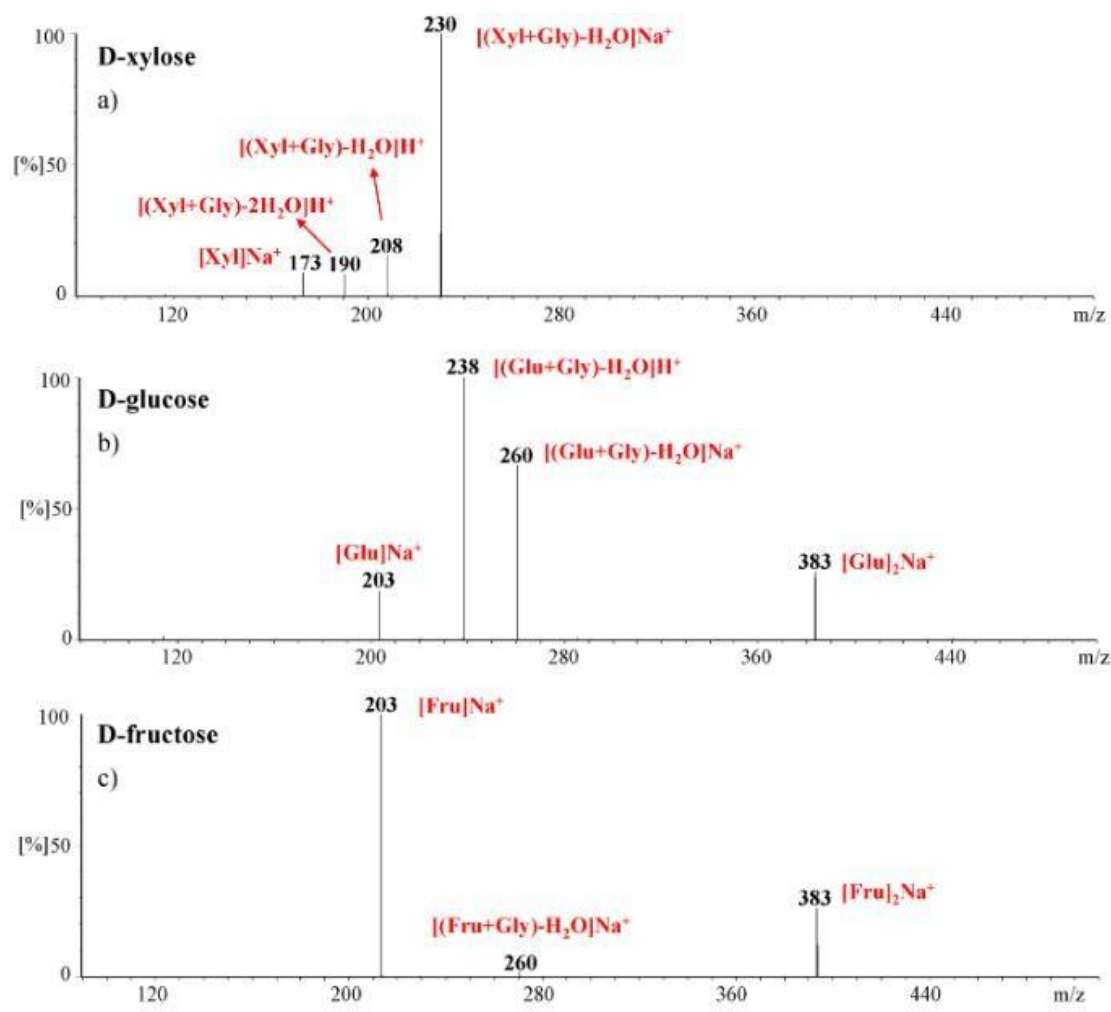


Figure 3. The ESI mass spectra of the solutions obtained by rinsing the solid precipitate generated by the ESI microdroplet deposition of: (a) 1 mL of 1:1 L-glycine/D-xylose 10^{-3} M $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ 3:1 solution; (b) 1 mL of 1:1 L-glycine/D-glucose 10^{-3} M $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ 3:1 solution; (c) 1 mL of 1:1 L-glycine/D-fructose 10^{-3} M $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ 3:1 solution.

In the L-glycine/D-xylose thin film (Figure 2a), the extensive formation of the MRPs, corresponding to the addition of the amino acid to the sugar with the concomitant removal of a water molecule, is evidenced by the protonated and sodiated ions at m/z 208 and m/z 230 respectively. An ionic

species corresponding to the loss of an additional water molecule can also be observed at m/z 190.

In the L-glycine/D-glucose system, similarly abundant protonated and sodiated ions were present at m/z 238 and m/z 260, corresponding to the first step reaction products of the Maillard reaction (Figure 2b).

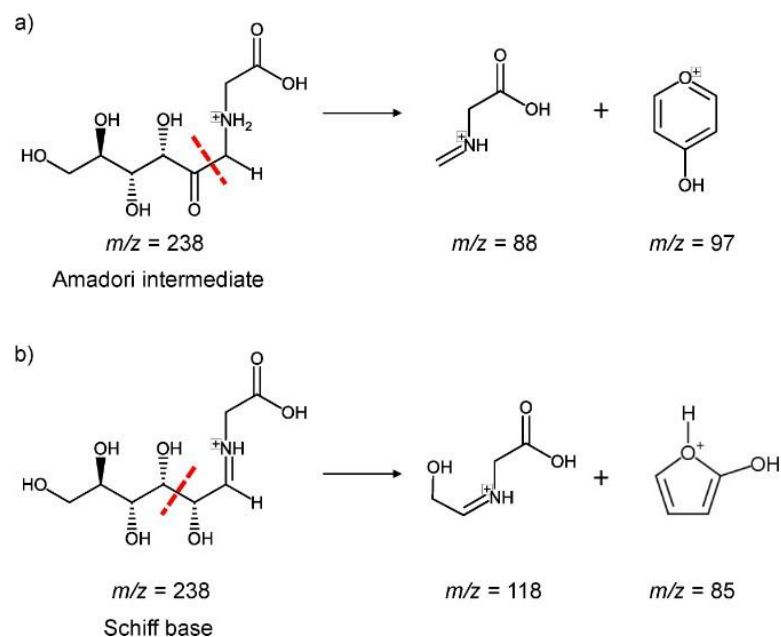
Conversely, only traces of the sodiated Maillard product at m/z 260 were observed with D-fructose (Figure 2c).

Moving to the L-lysine/D-xylose and L-lysine/D-glucose solutions, the MRPs are evidenced by the formation of the protonated species at m/z 279 and m/z 309 respectively (Figure 3a, b).

In these systems it is also evident that the addition of a second sugar molecule leading to the ionic products at m/z 411 and m/z 471 is due to the presence of two amino groups in the L-lysine structure. It is interesting to note that all the above ionic species, which are evidence of the Maillard reaction, are the most abundant ions in the ESI mass spectra of the aldose-containing systems. Moderate formation of the MRPs was observed when L-lysine was allowed to react with D-fructose, which, moreover, was limited to the addition of a single sugar molecule (Figure 3c).

Considering the absence of D-fructose addition product with L-glycine, it is reasonable to assume that its addition to L-lysine occurs in the more reactive ε -NH₂ group, confirming the lower reactivity of the α -amino group of amino acids with ketoses. In particular, the low formation of early-stage Maillard reaction products in D-fructose/amino acid systems has been attributed to the formation of advanced Maillard products and/or their easy degradation by the caramelisation reaction [21,22,23]. However, in the thin film systems containing D-fructose, the existence of these processes can be excluded, as demonstrated by the almost unaltered ionic signals of the starting reactant observed in the mass spectra of the rinsed precipitates (Figure 2c and Figure 3c).

The results of a recent mass spectrometric study by Xing et al [24] on the fragmentation pattern of the L-glycine/D-glucose adduct allow them to distinguish whether the amino acid/sugar addition products were characterised by the structure of the initially formed Schiff base or by the structure of its Amadori rearrangement isomer. The main diagnostic MS/MS fragmentations that distinguish the Schiff base from the Amadori intermediate are shown in Scheme 2.



Scheme 2. The most important diagnostic fragmentations of: (a) Amadori intermediate and (b) Schiff base.

The ions at m/z 88 and m/z 97, generated by the cleavage of the monosaccharide carbon chain at the C1-C2 position, were taken as diagnostic peaks for the presence of the Amadori product, whereas the ions at m/z 118 and m/z 85 were considered to be indicative of the formation of the Schiff base. Considering these results, the L-glycine/D-glucose adduct at m/z 238 was mass isolated and subjected to MS_n experiments in an ion trap mass spectrometer (Figure 4).

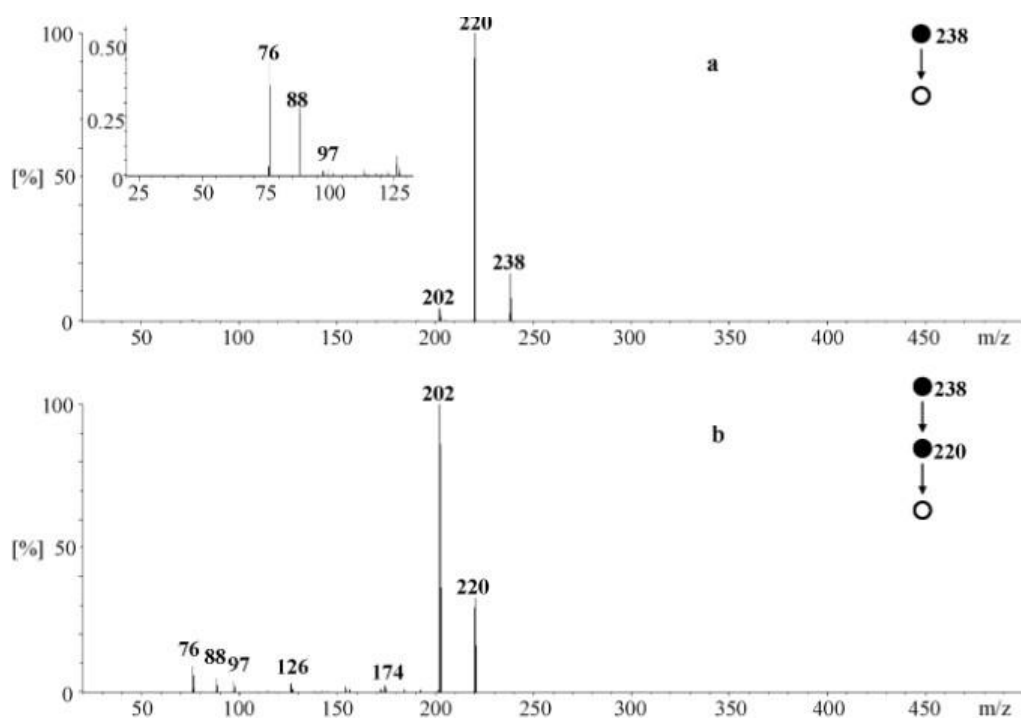


Figure 4. The ion trap MS² (a) and MS³ (b) CID spectra of the L-glycine/D-glucose adduct at m/z 238. The inset shows the diagnostic fragmentation peaks in the m/z 25–130 interval of MS².

The protonated species at m/z 238 dissociate predominantly by releasing two water molecules, resulting in the formation of the fragment ions at m/z 220 and m/z 202 respectively (Figure 4a). Despite their high abundance in the CID mass spectrum, these ionic species are not useful in discriminating between the isobaric species mentioned above.

In contrast, the minor fragment ions at m/z 88 and m/z 97 are responsible for the formation of the Amadori intermediate at the expense of the Schiff base (Figure 4a).

The same product ions are also present in the CID mass spectrum of the dehydration product at m/z 220, obtained from the precursor ion at m/z 238 by a second cycle of isolation/fragmentation (Figure 4b).

The absence of any Schiff base diagnostic fragments clearly indicates the predominant formation of the Amadori rearrangement product from the MR between L-glycine and D-glucose carried out in the thin film.

Similar experiments were carried out on the sodiated L-glycine/D-glucose product at m/z 260.

There was no significant evidence of isomer discrimination. Analogous results were obtained for the corresponding L-glycine/D-xylose product ions at m/z 208, also showing the diagnostic fragment at m/z 88, whereas for all L-lysine/sugar systems, the cleavage of the amino acid backbone predominates over the cleavage of the monosaccharide carbon chain and no diagnostic fragments were detected for correct ion assignment. However, it is reasonable to assume that also in the L-lysine systems the Schiff base evolves to form the Amadori intermediate with D-glucose and D-xylose aldose sugars.

MR is a thermal process mainly affected by the reaction temperature, the effect of the desolvation gas temperature of the ESI source, which promotes solvent evaporation in the deposited thin film, was investigated in detail.

The temperatures of the desolvation nozzle were varied from 50 to 350 °C and the corresponding actual temperatures of the collected thin film were about 30% of the set desolvation gas temperature (i.e. a desolvation gas temperature of 200 °C corresponded to a thin film temperature of about 60 °C).

Figures 5 and 6 show the sum of the relative intensities of the Maillard products with respect to the unreacted sugar and amino acid as a function of the actual thin film temperatures.

The values shown are the average of three different measurements. Due to the low reactivity of D-fructose, these experiments were limited to D-xylose and D-glucose.

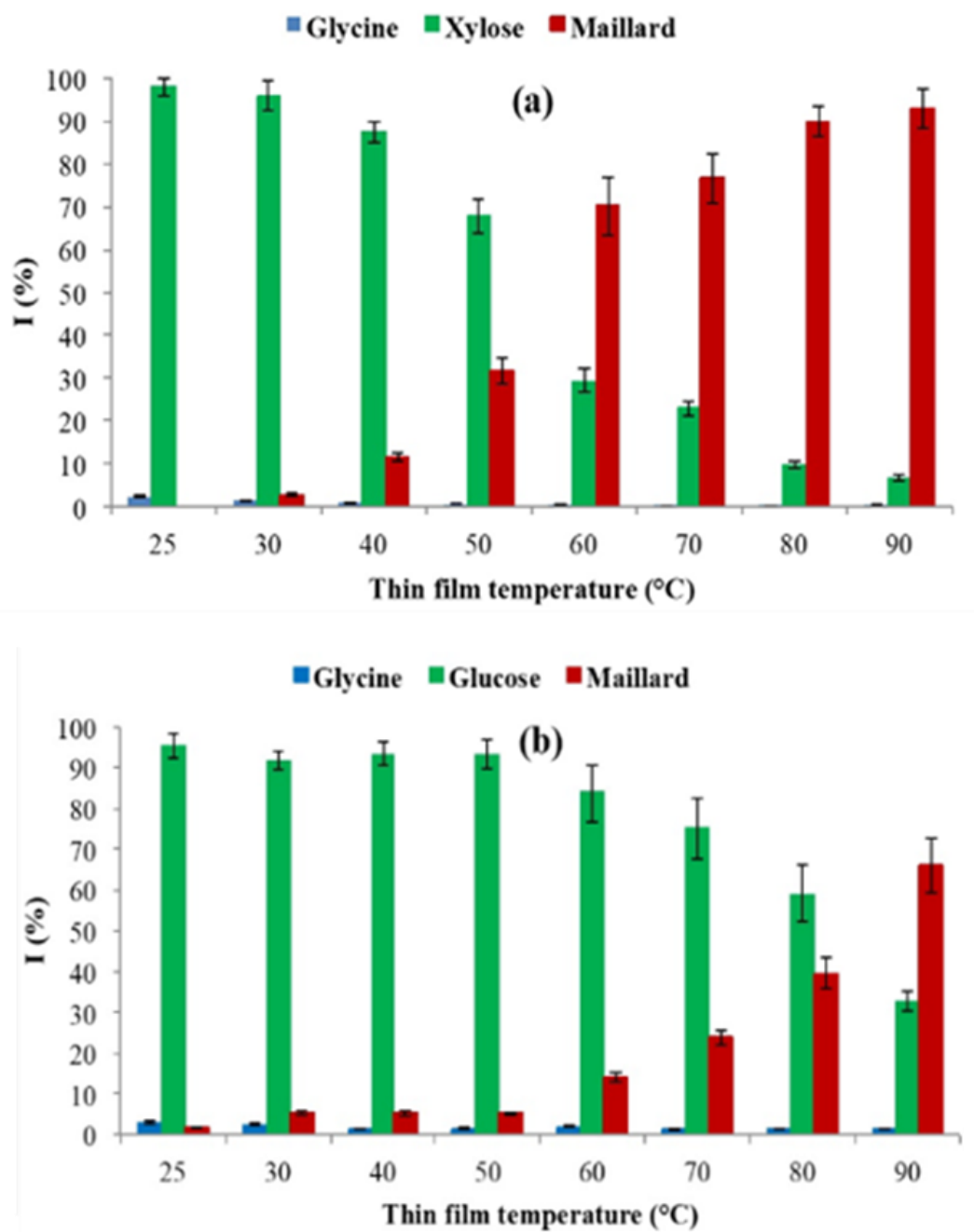


Figure 5. The reactants and Maillard reaction products (%) versus thin film temperatures (°C): (a) L-glycine/D-xylose mixtures and (b) L-glycine/D-glucose mixtures.

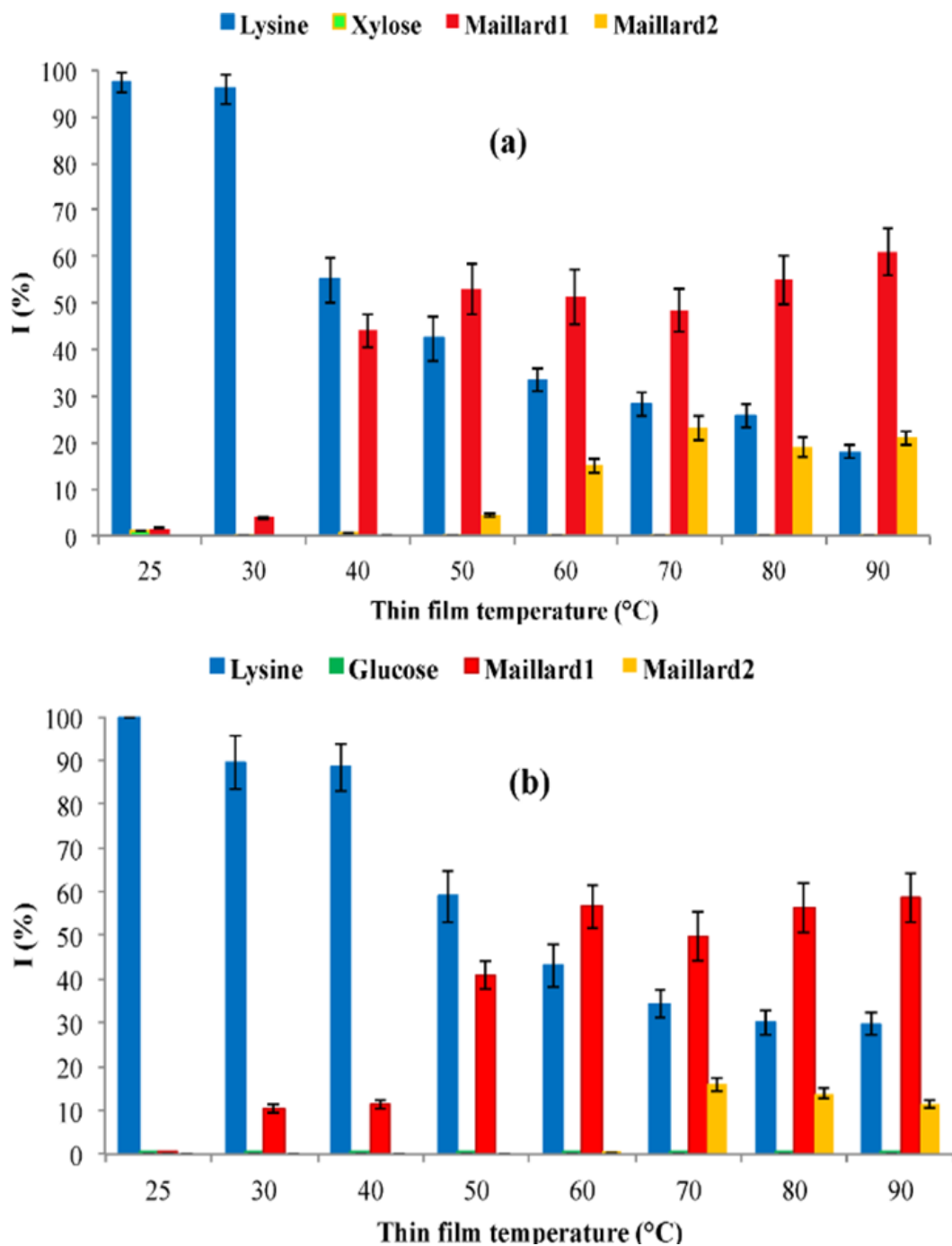


Figure 6. The reactants and Maillard reaction products (%) versus thin film temperatures °C. (a) L-lysine/D-xylose mixtures and (b) L-lysine/D-glucose mixtures.

As emphasised in the previous paragraph, L-glycine is hardly ionised under ESI conditions. In the reaction mixtures containing this amino acid, the abundances of the unreacted species were derived from the decrease of the sodiated sugar ions.

Conversely, the consumption of reactants in the L-lysine system is measured by the decrease of its protonated species, since L-lysine is efficiently ionised by protonation to its basic ϵ -amino group.

This discrepancy does not allow, at this stage, a comparison of the reactivity of the two amino acids, although it is possible to draw some considerations regarding the different reactivity of the two sugars with respect to the same amino acid.

In the L-glycine/D-xylose mixture (Figure 5a), the MRP (red bars) predominates over the unreacted species (green bars) at a temperature of at least 60°C and represents almost 90% of the total ionic current at a temperature of 80°C.

A temperature of about 90 °C is required to obtain a MRP ion intensity of 70% in the reaction with D-glucose (Figure 5b).

It seems evident that D-xylose is more reactive towards L-glycine than D-glucose. Moving to the L-lysine systems, the two addition products have been termed Maillard 1, assuming that the first sugar molecule is bound to the ϵ -NH₂ group, and Maillard 2, with an additional sugar molecule bound to the α -NH₂ group.

Higher temperatures than those observed for D-xylose catalyse the addition reaction of D-glucose to L-lysine.

The Maillard 1 reaction products reach a maximum even at 50 °C in the case of D-xylose (Figure 6a), whereas a similar product distribution can be achieved in the D-glucose solution at around 60 °C (Figure 6b).

The addition of the second sugar molecule started at a temperature of around 50 °C for D-xylose and at least 70 °C for D-glucose.

4.3 Thin Film Maillard Kinetics Relative to Bulk Solution

The kinetics of the observed first step of the Maillard reaction in the thin film was studied by extending the life of the thin film containing a fixed amount of the two reactant molecules over time. For this purpose, the pure solvent was infused for different periods of time after the 1:1 10^{-3} M amino acid/sugar mixture had been deposited on the collecting surface for 5 min.

In this way, the thin film was continuously renewed without adding new reagents and ensuring a constant reaction volume.

Using this procedure, it is not possible to estimate an absolute value of the reaction kinetic constant, as the actual thin film volume and reactant concentration cannot be known.

However, relative reaction rates can be obtained by comparing the kinetic data measured in the different sugar/amino acid systems in a self-consistent kinetic model [25].

The bimolecular Maillard reaction was considered as a second order reaction, as usually reported in the literature, although some kinetic studies claim that this process is a zero and first order reaction under certain synthetic conditions [26,27].

Assuming that the concentration C of a reactant species can be described by its ion abundance, the value of $1/C$ was plotted against reaction time, yielding the best-fit lines shown in Figure 7.

The slopes of the best-fit lines were then used to measure the ratio between the kinetic constants of the different sugar/amino acid systems, assuming that:

$$\frac{k_{system1}}{k_{system2}} = \frac{slope_{system1}}{slope_{system2}}$$

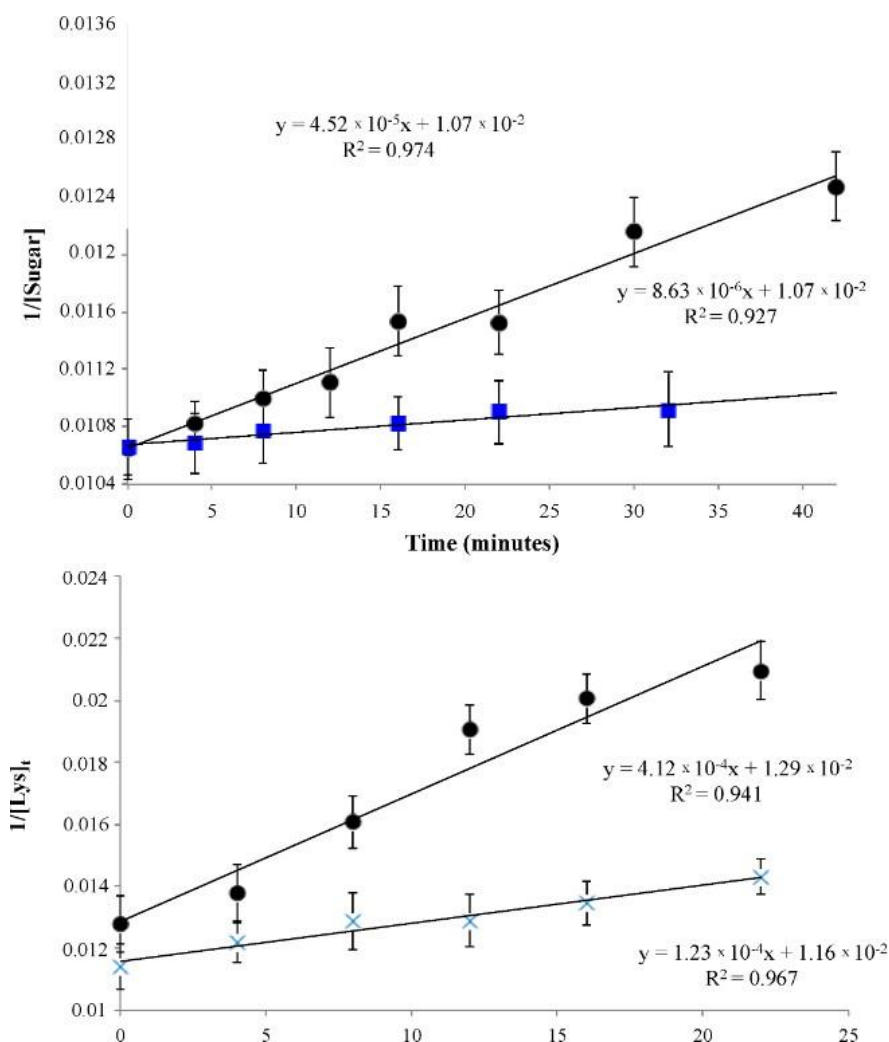


Figure 7. The kinetic plots of the second-order thin film Maillard reaction for the (a) L-glycine/sugar systems and (b) L-lysine/sugar systems.

Due to the different ionisation efficiencies of the amino acids, the best-fit lines of the D-xylose and D-glucose reactions against L-glycine were constructed by following the relative intensity of the sugar ions, whereas for the reactions involving L-lysine the plot was based on the relative intensity decrement of the L-lysine ions.

Since the kinetic data of the different mixtures have to be compared under the same experimental conditions, and in the case of L-lysine it is necessary to avoid the presence of the competing addition of the second sugar molecule, the kinetics of all reactions were studied at a thin film temperature of 40 °C (desolvation gas temperature of 100 °C).

This temperature also represents the higher temperature at which the extensive conversion of reactants to products during initial deposition is prevented.

The slopes of the lines relevant to the reactions involving D-xylose were significantly higher than those found for the D-glucose systems. In particular, the reactions of D-xylose with L-glycine and L-lysine were about five and three times faster, respectively, compared to the reaction with D-glucose. These results are consistent with the well-recognised reactivity of different sugars with amino acids observed in solution, which generally showed a faster Maillard reaction with pentose sugars than with hexose [28].

Although the ionic species used to construct the kinetic plot for the two amino acid solutions were different, it is reasonable to conclude from the relative slopes obtained that L-lysine is more reactive than L-glycine with both sugars tested by a factor of about 10. This acceleration factor underlines the greater reactivity of the ϵ -amino group of the amino acids with respect to the α -amino group.

To determine the rate acceleration factors (RAF) [29] of the Maillard reaction carried out by microdroplet thin film deposition with respect to the same reaction in bulk, 10 mL of the 3:1 H₂O/CH₃OH 10⁻³ M amino acid/sugar 1:1 solution was heated under reflux for 1 h. No products were detected in bulk at 40 °C. To measure a lower limit of the RAF, the bulk reactions were therefore tested with 10⁻¹ M solutions of the reactants and the temperature raised to 100 °C. Interestingly, by using stronger synthetic conditions, no reaction products were observed for the L-glycine/D-glucose system. Conversely, small amounts of MRPs were observed for the reactions of D-xylose with L-glycine and L-lysine and D-glucose with L-lysine. Aliquots of the reaction mixtures were taken at different reaction times and the kinetics of the reactions were followed by analysing the product concentration under the same mass spectrometric ESI conditions used to study the kinetics of the thin film reactions (Figure 8).

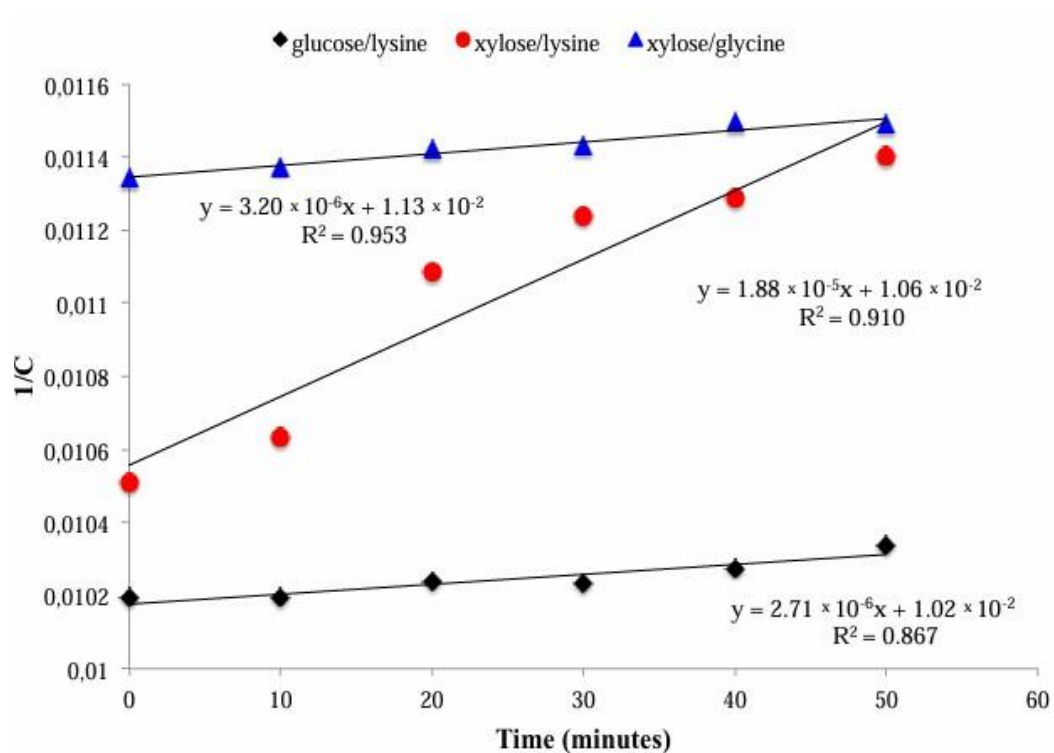


Figure 8. Kinetic plots of the reactions performed in bulk.

The whole slopes measured from the kinetic plots of the thin film and bulk reactions with the derived RAF are reported in Table 1.

Table 1. The kinetic parameters and derived RAF for the investigated thin film and bulk reactions.

System	Slope (Thin Film)	Slope (Bulk)	RAF
D-xylose/L-glycine	4.52×10^{-5}	3.20×10^{-6}	14.1
D-xylose/L-lysine	4.12×10^{-4}	1.88×10^{-5}	21.9
D-glucose/L-glycine	8.63×10^{-6}	-	∞
D-glucose/L-lysine	1.23×10^{-4}	2.71×10^{-6}	4 5.4

Taking into account that the bulk reactions were conducted at a higher solution temperature than that used for the thin film (100 °C versus 40 °C), it is evident that the strong acceleration of the first dehydration step of the Maillard reaction in the thin film environment.

The reactions of D-xylose toward L-glycine and L-lysine were accelerated by factors ranging from 14 to 22. Moreover, it is interesting to underline that D-glucose, which was less reactive than D-xylose toward the amino acids used both in solution and in the thin film, showed a high thin film RAF that, in the case of L-lysine, was at least double with respect to that observed with D-xylose (45.4).

4.4 Conclusion

The MR is one of the landmark processes in food chemistry, responsible for the flavour and nutritional value of food products. In recent years, the Maillard reaction has shown great potential in the preparation of flavourings for processed foods, which are needed to formulate more acceptable products for consumers. Here we have demonstrated the possibility of synthesising an almost pure Amadori compound, taking advantage of the accelerated reactivity in the confined volume of thin films formed by ESI microdroplet deposition.

The kinetic studies of the thin film reactions of D-xylose and D-glucose with L-glycine and L-lysine show strong acceleration factors compared to the same reactions in bulk. The glycosylation of L-glycine in the thin film environment is carried out under mild synthetic conditions where the same reaction is not observed in solution. This small-volume procedure may represent a useful synthetic method to obtain a Maillard reaction product that can be scaled up for industrial application.

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5. Sulfuric Acid Catalyzed Esterification of Amino Acids in Thin Film

5.1 Introduction

Amino acids are the building blocks of proteins, hormones, and neurotransmitters in living organisms. Amino acid deficiencies can be treated by dietary supplementation, but amino acid drugs generally have low bioavailability due to low intestinal permeability, extensive metabolism, and rapid hepatic clearance [1-2].

In addition, the presence of a carboxyl function, an amine group, and possibly aromatic, amine, carboxyl, and hydroxyl groups in a single molecule provides a variety of opportunities to tailor the final properties of the drug. For this reason, amino acid esters are widely used as amino acid precursors in the food and pharmaceutical industries [3-8].

Amino acid esters are used in food industries to improve the flavor of foods and extend the shelf life of products.

Other uses include fortifying foods with essential and balancing amino acids for diets, infant formulas, low-calorie foods, spices, and adding flavor and aroma to foods [9].

There is a relationship between the configuration of the amino acid itself and the perceived taste; this phenomenon appears to be due to stereospecific adsorption on taste receptors on the surface of the tongue. Glutamic acid is one of the most widely used amino acids in food; in fact, both its sodium salt and ester forms are commonly used as effective flavor enhancers, especially to improve the taste of meat and vegetable dishes.

Dipeptides formed from sweet amino acids are generally tasteless, while peptides containing bitter amino acid residues all taste bitter. The start in this field was made with the discovery of the methyl ester of L-aspartyl-L-phenylalanine, which tastes sweet, sweeter than sucrose by a factor of 150 [9].

In addition, the amino acids and their esters have been patented for use as antioxidants.

Alkyl esters (CH_3 , C_2H_5) of these amino acids are readily soluble in oils and fats, do not cause unpleasant odours or discoloration when heated, and can thus be used as antioxidants and emulsifiers for food products.

Finding faster and cheaper ways to synthesize amino acid esters can be a significant advantage for the food industry.

For example, the economical synthesis of the methyl ester of L-phenylalanine is important for the production of one of the most widely used synthetic sweeteners, aspartame.

Amino acid esters are also used in polymer synthesis. For example, polyamino acids are usually obtained by polymerization of N-carboxyanhydrides, which are synthesized by the action of carbonyl chloride on amino acids [9].

Their backbone structure is similar to that of proteins, but they contain only one type of amino acid. Glutamic acid has been used not only in the food industry, but also in the form of polyglutamates as coatings for artificial leather. In the industry we find the application of two types of polymers based on γ -methyl glutamate, unmodified PGS (trade name PLG) and modified PGS (PLG-N).

A very important property of polyamino acids is their non-toxicity to body tissues. Of great interest is the production of polymers with biological, antimicrobial and anti-inflammatory activity. In a recent study, the ability of methyl esters of amino acids to possess antimicrobial activity was investigated [10].

Methyl esters of different amino acids have antimicrobial activity against gram-negative and gram-positive bacteria: especially *E. coli* and *S. aureus*.

Amino acid esters also find application in pharmacology mainly as prodrugs.

Amino acid drugs generally have low bioavailability due to low intestinal permeability, extensive metabolism in the intestine, and rapid elimination by the liver [11].

Transient modification of the physicochemical properties of the molecule by chemical derivatization is the approach of choice for such drugs.

These structural changes increase the hydrophobicity of the molecule and decrease its ability to form hydrogen bonds, which has a positive effect on the stability of the prodrug against metabolic enzymes and further improves oral bioavailability [12].

For example, a recent study investigated and evaluated the ability of L-tyrosine methyl ester (L-Tyr-Ome) to produce an antinociceptive effect in mice [13].

These results suggest that L-Tyr-Ome-induced antinociception is mediated by central δ -opioid receptors and the noradrenergic system.

In addition, similar derivatives of L-tyrosine have been proposed for the treatment of Parkinson's disease [14].

In addition, conventional peptide synthesis and protein analysis protocols often take advantage of amino acid esterification to protect the -COOH group [15].

Amino acid esters are synthesized using a variety of different catalysts, ranging from classic mineral acids such as HCl and H₂SO₄, to ionic liquids, zeolites, microwaves, and enzymes [16-26].

Rare-earth doping of solid superacids has been used to prepare various ester compounds, and Nd doping was recently employed for aromatic amino acid esterification [27].

The esterification reaction is usually carried out in the presence of the corresponding alcohol as an organic solvent, and continuous removal of water is required to advance the reaction equilibrium.

In addition, the esterification of amino acids is much more difficult than that of ordinary carboxylic acids due to their zwitterionic structure, and reaction yields are often low due to the concomitant alkylation of the amine group, which requires tedious N-protection, esterification, and deprotection procedures.

The goal of this work was to exploit the properties of microdroplets to speed up the reaction compared to the same process in bulk.

The carboxylic acid esterification reaction, similar to other reactions that involve the loss of a water molecule, can take advantage of the rapid evaporation of the solvent that occurs in confined volume systems.

5.2 Results and Discussion

Thin film amino acid esterification with methanol was studied by spraying onto a target stainless-steel surface the solutions of five different amino acids (L-alanine, L-phenylalanine, L-tyrosine, L-lysine, and L-glutamic acid) in the presence of different amounts of H_2SO_4 as a mineral acid catalyst.

The selected reactants were chosen as being representative of differently side chain substituted amino acids, having additional alkyl, aromatic, amino, and COOH functional groups.

Initial millimolar solutions were prepared by dissolving L-amino acids in 1:1 $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ solvent mixtures and adding an inorganic acid catalyst (H_2SO_4 , HCl or HNO_3).

The use of buffers was avoided to prevent any interference with mass spectrometric analysis.

The resulting solutions were infused into the ESI source of the instrument, and the ionic composition of the microdroplets was analyzed online before performing the deposition experiments.

The mass spectra of the starting solutions containing H_2SO_4 catalyst at $\text{pH} = 2$ compared with the spectra of the thin film recovered products for all the investigated aminoacids are reported, are shown in Figures 2-4.

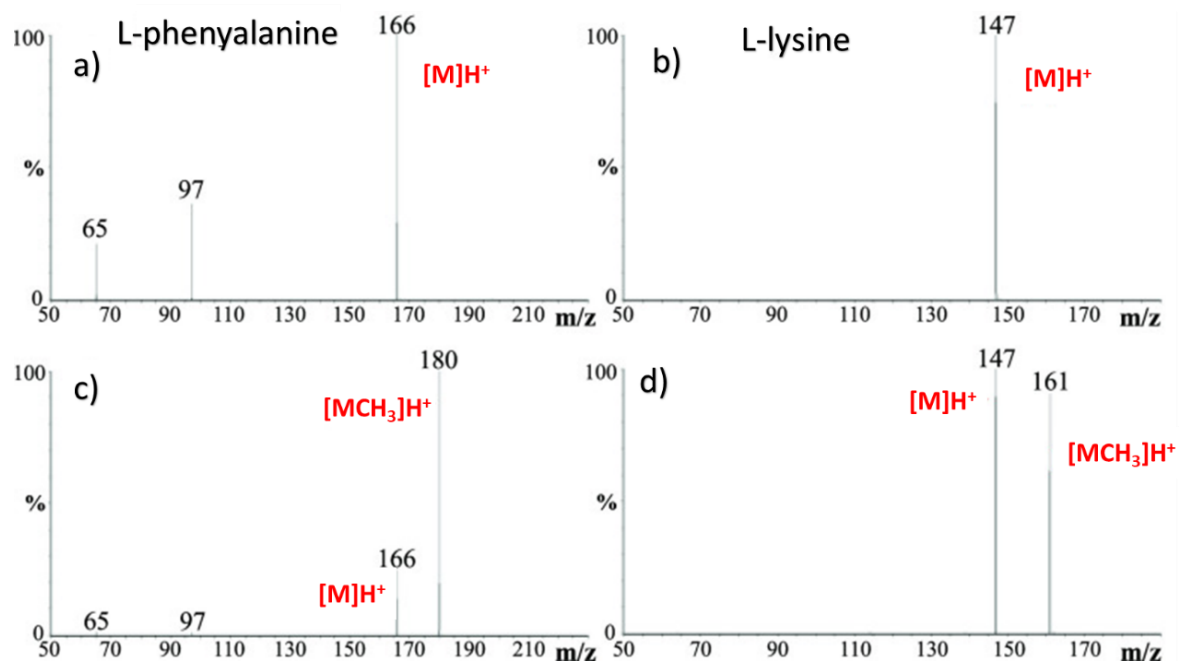


Figure 1. Positive ESI mass spectra of the starting solutions containing (a) L-phenylalanine and (b) L-lysine dissolved in a 1:1 H_2O , H_2SO_4 ($pH=2$)/ CH_3OH mixture at a concentration of 10^{-3} M. Positive ESI mass spectra of the thin film products formed from (c) L-phenylalanine and (d) L-lysine systems. Protonated amino acids and the corresponding methyl esters are indicated as $[M]H^+$ and $[MCH_3]H^+$, respectively. Ions at m/z 65 and 97 refer to $[(CH_3OH)_2]H^+$ and $[(CH_3OH)_3]H^+$ methanol adducts, respectively.

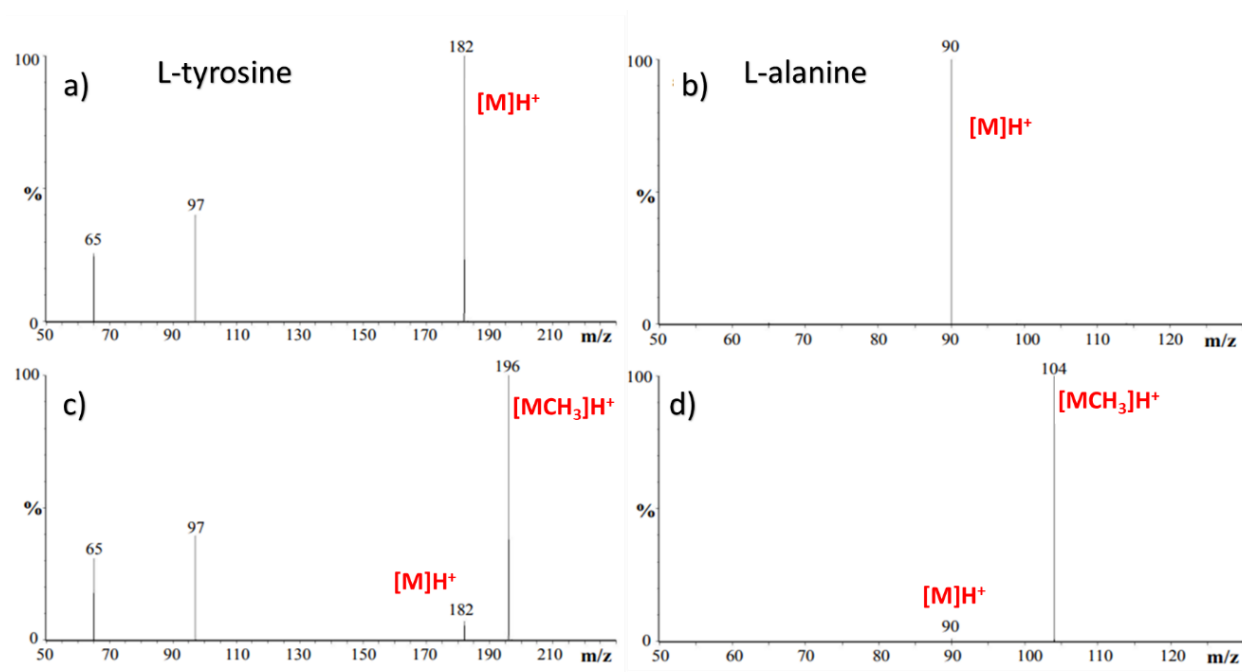


Figure 2. Positive ESI mass spectra of the starting solutions containing (a) L-tyrosine and (b) L-alanine dissolved in a 1:1 H_2O , H_2SO_4 ($pH=2$)/ CH_3OH mixture at a concentration of 10^{-3} M. Positive ESI mass spectra of the thin film products formed from (c) L-tyrosine and (d) L-alanine systems. Protonated amino acids and the corresponding methyl esters are indicated as $[M]H^+$ and $[MCH_3]H^+$, respectively. Ions at m/z 65 and 97 refer to $[(CH_3OH)_2]H^+$ and $[(CH_3OH)_3]H^+$ methanol adducts, respectively.

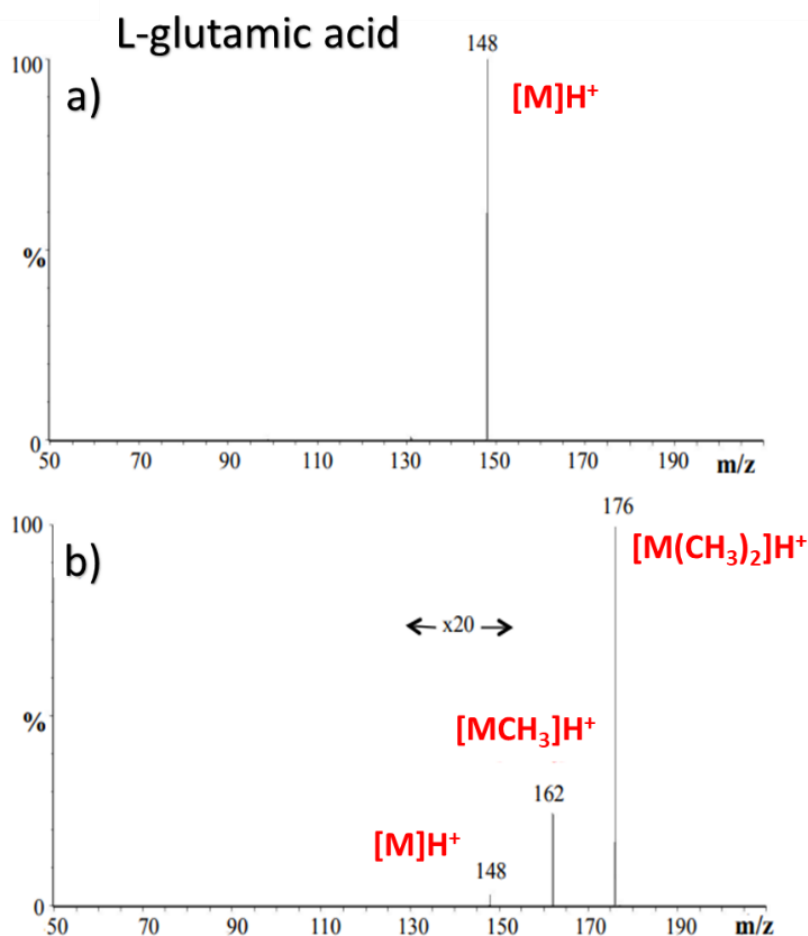


Figure 3. (a) Positive ESI mass spectrum of the L-glutamic acid 1:1 H_2O , H_2SO_4/CH_3OH 10^{-3} M starting solution at $pH=2$. (b) Positive ESI mass spectra of the thin film products formed from (b) L-glutamic acid systems.

As shown in Figure 1a, b, the ESI positive mass spectra of the H_2SO_4 L-phenylalanine and L-lysine starting solutions at $pH=2$ are dominated by the amino acid MH^+ ion at m/z 166 and 147, respectively.

Intense ionic signals corresponding to methanol protonated clusters are also present in the spectrum of L-phenylalanine, whereas the presence of an additional $\epsilon-NH_2$ basic group in L-lysine strongly favours the formation of its protonated species.

No ionic species attributable to the amino acid esterification were observed, thus demonstrating that no reaction occurs in the microdroplets before their deposition onto the solid surface.

The deposition of the ESI microdroplets onto the stainless-steel surface generates a stable thin film from which a yellow precipitate separates during the reaction time (typically 50 min).

Besides the amino acid MH^+ ions, the ESI mass spectra of the precipitates rinsed with the H_2O/CH_3OH solvent mixture show additional ions at m/z 180 and 161 formally corresponding to the addition of a methyl group to L-phenylalanine and L-lysine, respectively (Figure 1c, d).

Similar results were obtained for all the other amino acid systems studied (Figure 2). In the case of L-glutamic acid, the simultaneous formation of mono- and dimethylated products was observed (Figure 3), probably due to the esterification of its two $COOH$ functional groups.

Analogous thin film reaction experiments were also carried out by depositing the microdroplets produced in the negative ESI ion mode or with no potential applied to the ESI needle. For direct comparison, the rinsed precipitate was always analysed in positive ion mode, giving good signals for both the free and esterified amino acids.

To verify that the observed products correspond to the methyl ester of the amino acid $-COOH$ group, the CID mass spectra of the thin film products were compared with those of commercially available standards (Figures 4-7).

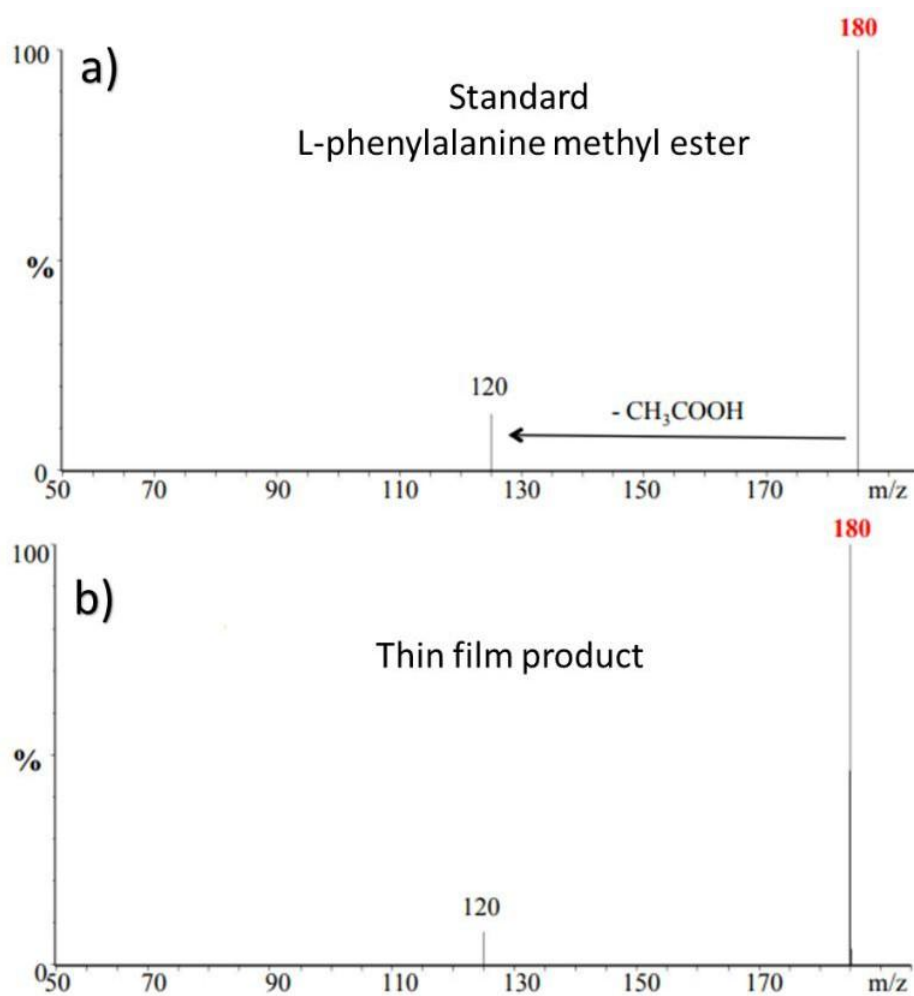


Figure 4. a) CID mass spectrum of standard L-phenylalanine methyl ester. b) CID mass spectrum of the thin film reaction product.

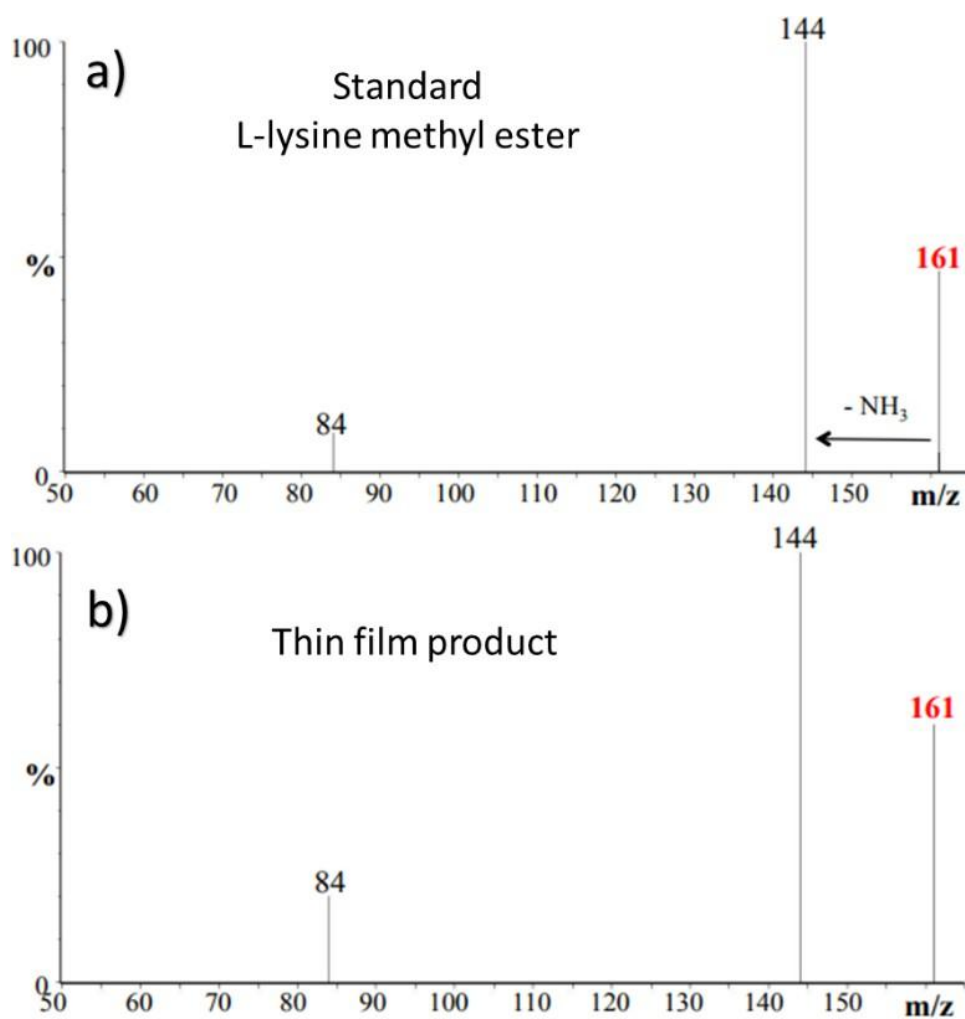


Figure 5. a) CID mass spectrum of standard L-lysine methyl ester. b) CID mass spectrum of the thin film reaction product.

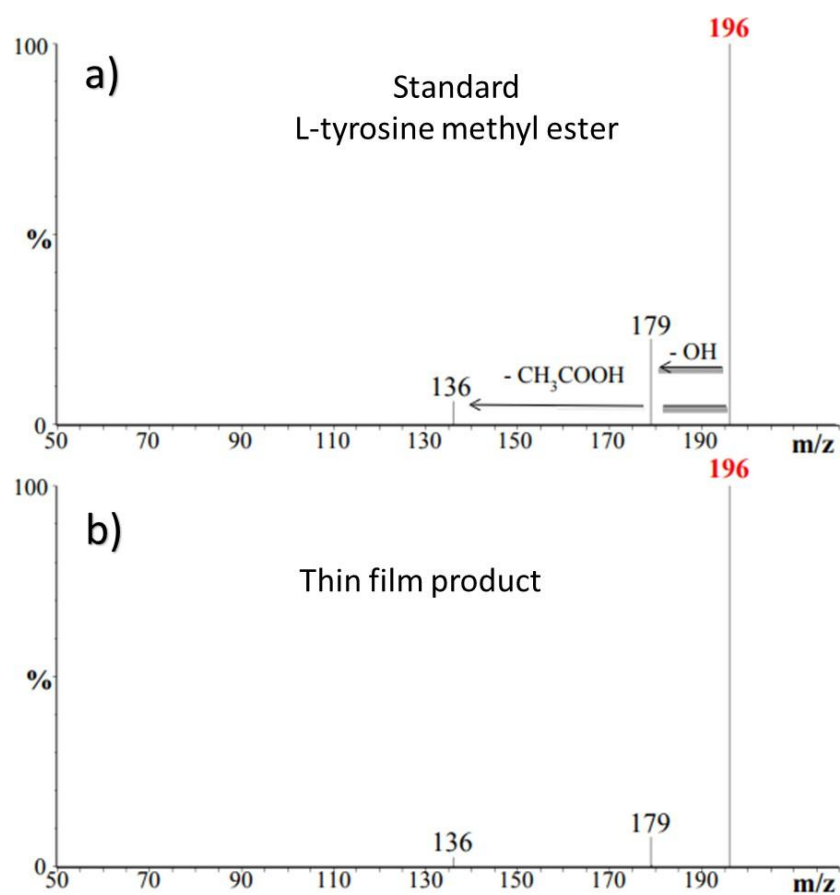


Figure 6. a) CID mass spectrum of standard L-tyrosine methyl ester. b) CID mass spectrum of the thin film reaction product.

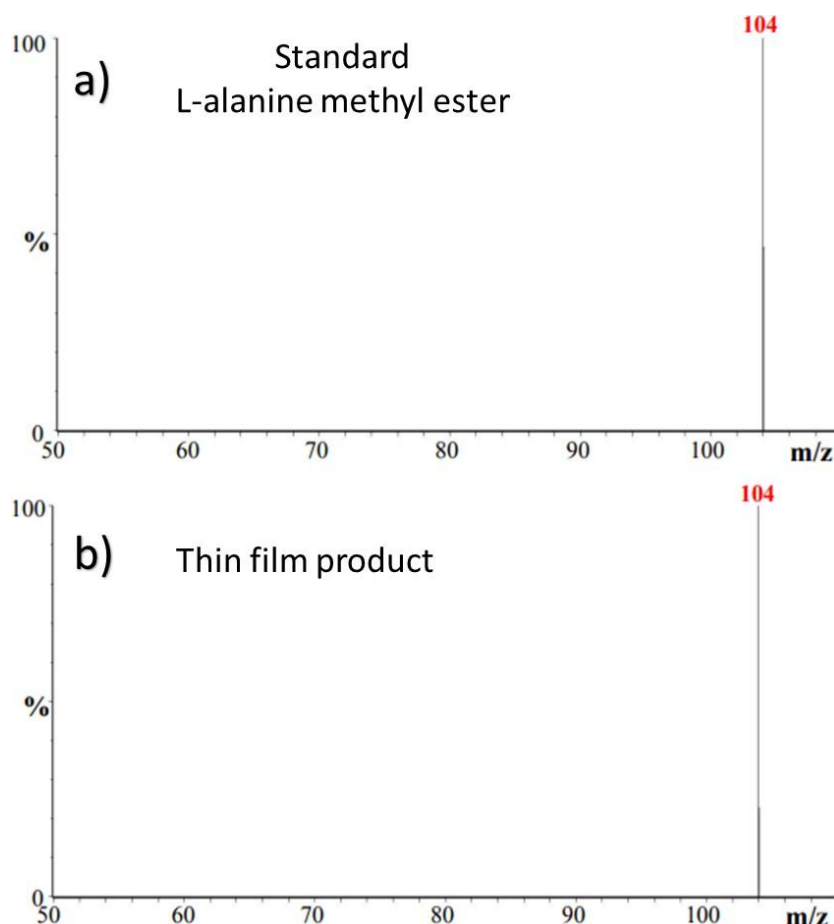


Figure 7. a) CID mass spectrum of standard L-alanine methyl ester. b) CID mass spectrum of the thin film reaction product.

Apart from the methyl ester of L-alanine, which does not fragment upon collision with Ar (Figure 7), and the methyl ester of L-lysine, which fragments after loss of one NH_3 molecule (Figure 6), the loss of a CH_3COOH moiety appears to be the peculiar fragmentation, indicating esterification of the COOH group. The CID mass spectra of the products derived from the thin-film reactions showed these fragmentations, all of which could be superimposed on those of the corresponding methyl esters of the standard amino acid.

In the case of L-glutamic acid, the CID mass spectrum of the standard L-glutamic acid 5-methyl ester (Figure 8a), corresponding to the amino acid esterified on the side chain COOH group, is characterised by the loss of methanol and HCOOH , whereas the monoester obtained from the thin film

reaction shows, in addition to these fragmentations, the loss of a water molecule (Figure 8b).

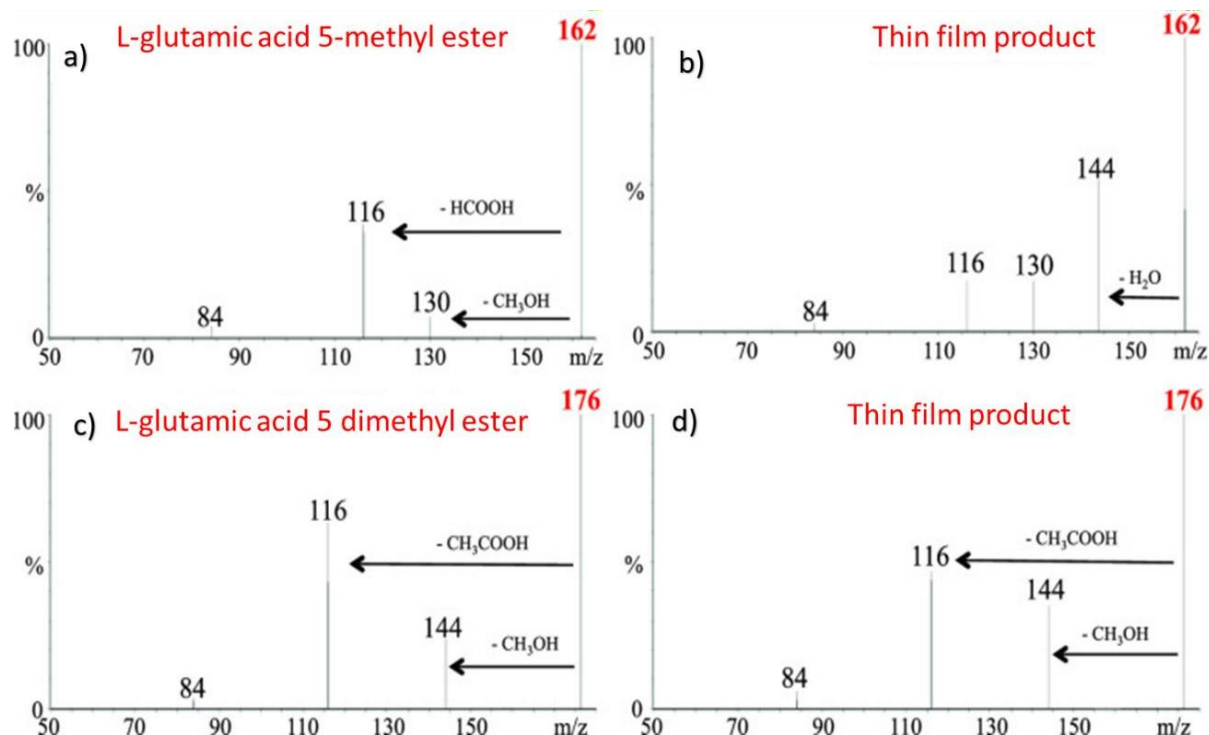


Figure 8. (a) CID mass spectrum of the standard compound l-glutamic acid 5-methyl ester. (b) CID mass spectrum of the l-glutamic monomethyl ester product formed by the reaction in the thin film. (c) CID mass spectrum of the standard compound l-glutamic dimethyl ester. (d) CID mass spectrum of the l-glutamic dimethyl ester product formed by the reaction in the thin film. The m/z ratios of the protonated parent species are indicated in red. Major fragment ions are highlighted with black arrows.

The losses of water and formic acid are the characteristic fragmentations of l-glutamic acid (Figure 9) and it is therefore reasonable to assume that the monoesterification of L-glutamic acid in the thin film reaction occurs at both amino acid COOH moieties. The CID of standard L-glutamic acid dimethyl ester can be superimposed on that of the ionic species derived from the thin film reaction (Figure 8c, d), showing the common loss of CH₃COOH and the small loss of methanol characteristic of esterification of the COOH side chain group.

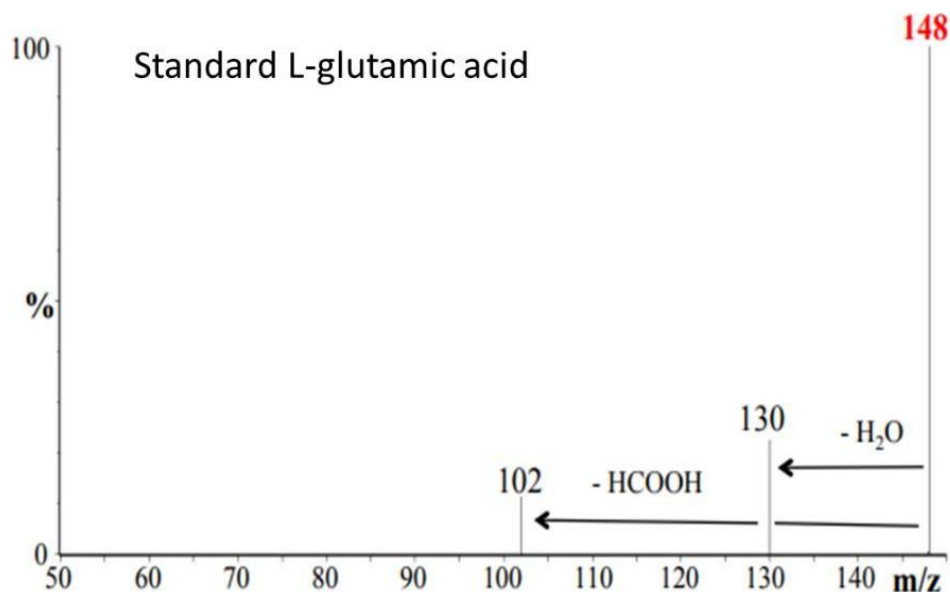


Figure 9. CID mass spectrum of standard L-glutamic acid.

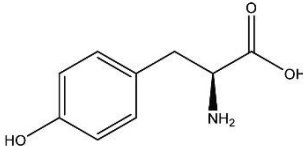
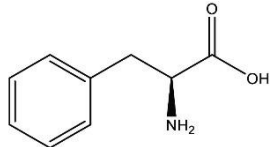
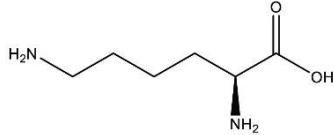
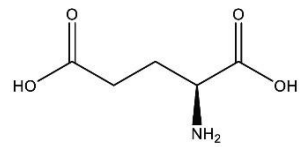
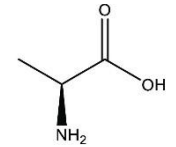
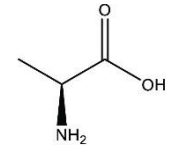
By measuring the conversion ratios (CRs) of all the amino acid thin film systems studied, summarised in Table 1, a first estimate of the efficiency of the esterification reaction observed was obtained.

Very low conversions were measured for the less acidic solutions at pH=2.7, whereas conversions of around 80-90% were measured for most of the amino acids at pH=2, with the exception of L-lysine, whose CR is around 50% in positive and negative ion mode and increases to 77.2% without the application of the cone voltage.

Each reported CR value represents the mean of three independent measurements. It is interesting to note that the ESI capillary voltage does not interfere with the reaction yields even in positive and negative polarities.

Moreover, the experiments carried out without the applied voltage always showed a slightly higher reaction efficiency. This effect could be explained by the formation of larger microdroplets whose deposition leads to the formation of a larger thin film volume, clearly visible even to the naked eye. Within this volume the molecules can continue to react until the solvent is completely dry.

Table1. Thin film conversion ratios (CRs), ionization correction factors (CFs), and reaction yields of the H_2SO_4 catalyzed amino acid esterification at different pH values and ESI cone voltage potentials^c.

Amino acid	pH	Cone Voltage kV	CR	Ioniz. CF	Yield%
	2.0	+4.5	92.3	2.1	43.9
	2.0	-3.5	85.3		42.6
	2.0	0	91.1		43.4
	2.7	+4.5	4.3		2.0
	2.0	+4.5	79.8	2.5	31.9
	2.0	-3.5	83.0		33.2
	2.0	0	99.2		36.7
	2.7	+4.5	0.23		0.1
	2.0	+4.5	47.2	2.3	20.5
	2.0	-3.5	55.8		24.2
	2.0	0	77.2		33.6
	2.7	+4.5	0.1		—
	2.0	+4.5	19.7 ^a	7.6	ne
			80.2 ^b	27.2	
	2.0	-3.5	17.5 ^a		ne
			82.4 ^b		
	2.0	0	19.6 ^a		ne
			80.3 ^b		
	2.7	+4.5	1.2 ^a		ne
			— ^b		
	2.0	+4.5	99.0	5.1	19.4
	2.0	-3.5	99.2		19.5
	2.0	0	99.1		19.4
	2.7	+4.5	—		—

It should be emphasised that the conversion ratios give only a rough estimate of the reaction yields, as they are strongly influenced by the ionisation efficiency of the reactants and products.

From this point of view, it is reasonable to assume that the real yields of the reaction should be better reproduced by the conversion ratio measured for amino acids with an additional basic group, as in the case of L-lysine, L-phenylalanine and L-tyrosine, whose basicity should hardly be influenced by the presence of the methyl substituent on the carboxyl function.

In order to test this assumption, the ionisation efficiency of each methyl ester with respect to the free amino acid was derived from the ratio of the intensities of the protonated ions of the ester (E) and the amino acid (A) measured in the ESI mass spectra of mixtures containing variable amounts of both molecules (termed $R_{E/A}$ experimental).

Calibration curves were then obtained by plotting these values against the theoretical ratio of mixed standard molecules (referred to as theoretical $R_{E/A}$).

The calibration curves of the amino acids studied are shown in Figure 10. The plots are all characterized by linear regression lines with R^2 exceeding 96%, and the ionization correction factors (CFs) derived were used to estimate the absolute yields reported in Table 1.

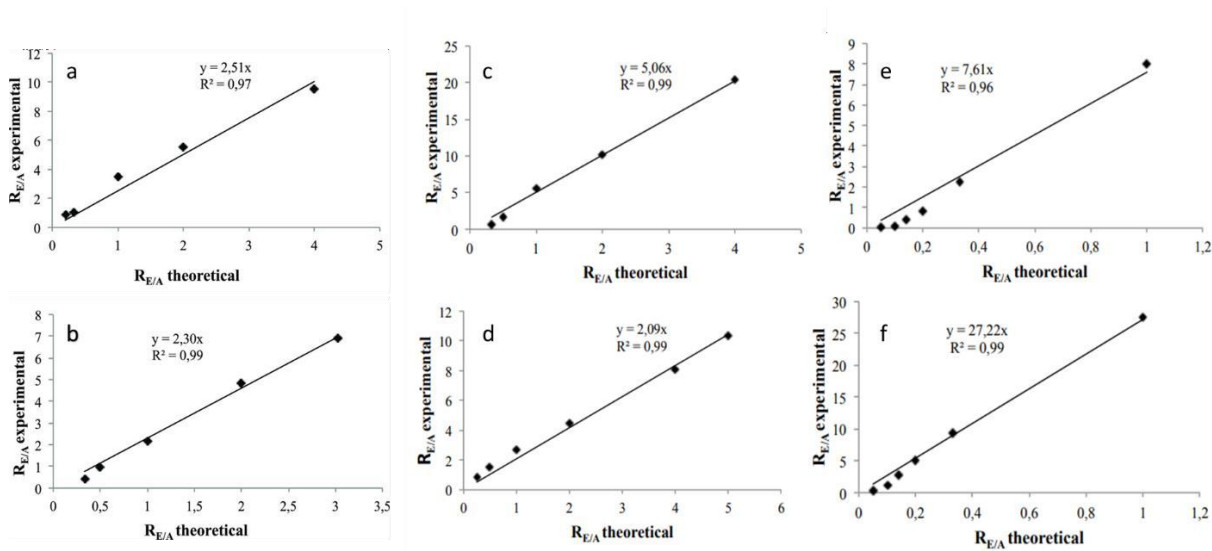


Figure 10. (a) L-Phenylalanine, (b) L-lysine, (c) L-alanina, (d) L-tyrosine, (e) L- acid glutamic and (f) L-acid glutamic dimethyl: calibration curves built by plotting the ratios of known amounts of standard amino acid (A) and the corresponding methyl ester (E) named $R_{E/A}$ theoretical and the protonated ester and amino acid ion intensities denoted as $R_{E/A}$ experimental. The linear regression analysis is reported in each graphic.

L-lysine, L-phenylalanine and L-tyrosine show ionisation efficiency correction factors of around 2, which leads to absolute reaction yields of around 40% for L-tyrosine and L-phenylalanine, while yields of around 25-30% can be estimated for L-lysine. For amino acids without additional basic groups, as in the case of L-alanine, or characterised by additional acid substituents, as in the case of L-glutamic acid, higher correction factors were measured, as expected.

The very low ionisation efficiency of free L-glutamic acid compared to its esters required the use of ester/amino acid ratios in favour of the amino acid in the ionisation efficiency calibration curves (Figure 10). This condition is in contrast to the ionic signal measured in thin-film experiments, where the free amino acid has a relative intensity that never exceeds 0.1% with respect to the sum of its esters (Figure 3).

For this reason, the CR of L-glutamic acid has not been corrected, since the values finally obtained contain a large error and are not representative of its actual esterification yields.

Moreover, absolute esterification yields of L-glutamic acid in line with those obtained for the other amino acids (around 50%) can be safely assumed, bearing in mind that when the ratio goes from 1:1 to 2:1 in favour of the ester, the peak relative to the protonated amino acid is no longer detectable.

5.3 Effects of the Thin Film Temperature

The parameters of the ESI source, such as the spray temperature, can strongly influence the volume and lifetime of the thin film obtained by microdroplet deposition [28].

In the instrumental setup used, the temperature of the thin film resulting from microdroplet deposition depends on the temperature of the nitrogen desolvation gas of the source block. This parameter was varied from 80 to 300 °C and the actual thin film temperature measured was about 50% of the set desolvation gas temperature.

Thus, the desolvation gas temperature of 150 °C used in the experiments described above corresponds to an actual thin film temperature of 70 °C.

The measured conversion ratios at nominal desolvation gas temperatures of 80, 150 and 300 °C for L-phenylalanine and L-lysine amino acid solutions taken as model system are given in Table 2. It is interesting to note that increasing the desolvation gas temperature from 150 to 300 °C leads to a drastic reduction in esterification yields, whereas the measured conversions are only slightly reduced by reducing the temperature to 80 °C. It is reasonable to assume that at higher temperatures the rapid desolvation of the microdroplets leads to the deposition of a smaller thin film volume which dries out rapidly in the solid state reactants and products, thus preventing the reaction from progressing. The optimum thin film reaction temperature is around 70°C.

Table 2. *Thin Film conversion ratios (CRs) measured at different temperatures.*

amino acid	desolvation gas temp (°C)	thin film temp (°C)	CR
L-phenylalanine	80	40	59.7
	150	70	79.8
	300	160	8.2
L-lysine	80	40	26.2
	150	70	47.2
	300	160	7.1

5.4 Effect of Acid Catalyst

Amino acid esterification was also tested using solutions containing HCl and HNO₃ as acid catalysts under the same conditions used for H₂SO₄ (pH=2).

Interestingly, no reaction products were observed, suggesting that not only pH but also the type of mineral acid used as catalyst influences the esterification reaction.

In solution, the ability of sulphuric acid and alkylsulphonic acid to promote esterification of the carboxyl group with alcohol is well known.

Indeed, it is reported that not only is the reaction mechanism catalyzed by acid, but the esterification is also facilitated by the alkylation of the carboxylate with sulphate or sulphonate esters formed by the reaction of H_2SO_4 with the alcohol used [29].

However, any attempt to find possible methyl sulphonate ions as reaction intermediates failed, even when the reaction mixtures were analysed using negative ESI ion mode.

5.5 Amino Acid Esterification in Thin Film versus Droplet Casting and Bulk

In order to determine the acceleration factors of the amino acid esterification observed in the thin film, the same reaction was carried out in bulk and in a static thick film under experimental conditions comparable to those used for the thin film experiments.

The bulk reactions were carried out by heating each amino acid solution at pH=2 for 1 h at 100 °C under reflux, while for the static thick film reactions a small volume of the same solutions was dropped onto a stainless-steel surface and dried in a thermostatic oven at 70 °C.

The mass spectra of the solutions taken at time zero and after the bulk and thick film reactions are completely superimposable and show only the amino acid MH^+ ions. No esterification reaction takes place in the bulk and thick film systems.

The H_2SO_4 catalyzed amino acid esterification reaction at pH=2 is peculiar to the thin film formed by ESI microdroplet deposition.

5.6 Conclusion

The esterification reaction of amino acids catalyzed by H_2SO_4 is accelerated by the rapid evaporation of the $\text{H}_2\text{O}/\text{CH}_3\text{OH}$ solvent mixture from the thin film formed by ESI microdroplet deposition.

The reaction is enhanced by the pneumatic spray of the ESI source, but is insensitive to the voltage applied to the ESI capillary and reaches its maximum efficiency at a thin film temperature of 70°C.

Critical parameters affecting the reaction outcome are microdroplet size and thin film volume and lifetime.

The use of sulphuric acid is crucial to observe the reaction, as no product was obtained using other strong mineral acids such as HCl and HNO₃.

The esterification takes place at temperatures and pH values at which the same reaction is not observed in bulk, and selective esterification of the COOH moiety has been demonstrated.

In accordance with the easier esterification of aromatic-substituted carboxylic acids compared to alkyl-substituted ones, L-tyrosine and L-phenylalanine, which share a common aromatic side-chain substituent, show higher yields among the amino acids studied.

The absolute yields of the reaction were evaluated by calibrating the ionisation efficiency of each amino acid with respect to its methyl ester, demonstrating that the conversion ratio, usually used to estimate the yield of microdroplet reactions, can be strongly influenced by the ionisation efficiency of the reagent and product.

Finally, the thin film synthesis procedure could be a model for other high efficiency esterification methods proposed in solution, which may occur under milder conditions when performed in confined volumes.

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6. Stereoselectivity in Electrosprayed Confined Volumes: Asymmetric Synthesis of Warfarin by Diamine Organocatalysts in Microdroplets and Thin Films

6.1 Is it Possible to use Microdroplets for Stereoselective Reactions?

Microdroplet reactions have received a lot of attention due to the specific nature of the reaction environment [1].

The formation of new carbon-carbon, carbon-oxygen and carbon-nitrogen bonds, dehydration, oxidation and reduction reactions have all been studied using microdroplets reactions [2-11].

However, a field not even explored is the use of electrosprayed microdroplet reactions in the synthesis of chiral compounds by asymmetric organocatalysis.

There are many examples of synthesis in microdroplets generated by ESI or DESI-based techniques, including the production of compounds with stereogenic centres [6,8] for which no stereoselectivity data are reported.

So, when we started our study, we wondered why the question of stereoselectivity had not been investigated before: do asymmetric reactions occur in ESI microdroplets? Does the stereoselectivity change compared to bulk conditions?

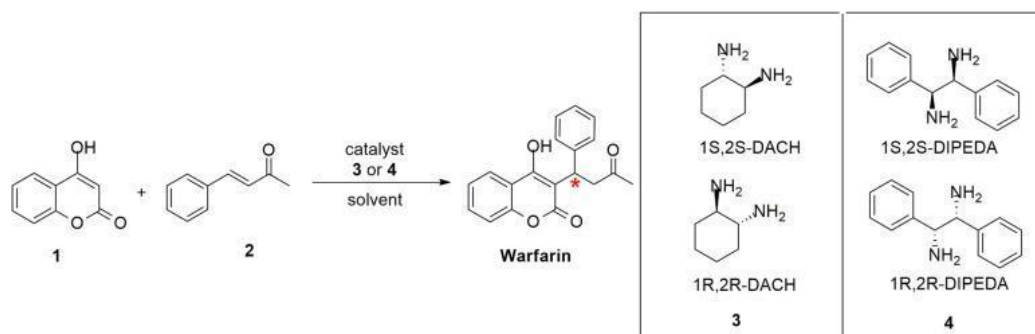
In this work, we have demonstrated that asymmetric organocatalyzed reactions can be carried out with stereoselectivity comparable to that obtained in bulk using the confined volumes of ESI microdroplets and the thin film formed by their deposition.

6.2 Results and Discussion

We chose to start exploring this field a model reaction: the asymmetric synthesis of warfarin, an anticoagulant vitamin K antagonist [13] with a stereogenic centre, which is marketed as a racemate, although the S enantiomer is more active than the R one [14-16].

The asymmetric synthesis of warfarin and its analogues is a model reaction that has been widely described in the evaluation of chiral amines and their derivatives as organocatalysts [17-21]. In this work, 1,2-diaminocyclohexane (DACH) **3** and 1,2-diphenylethylenediamine (DIPEDA) **4** were selected as chiral catalysts (Scheme 1) without the addition of acidic co-catalysts.

These diamines provide warfarin via the formation of an iminium ion with benzylideneacetone (**2**), followed by nucleophilic attack of 4-hydroxycoumarin (**1**).



Scheme1. Asymmetric synthesis of warfarin by diamine catalysts 1S,2S- or 1R,2R-DACH and 1S,2S- or 1R,2R-DIPEDA.

The first part of our work was devoted to optimising the conditions, moving from bulk to ESI microdroplet/thin film systems.

In previous studies was found that the reaction between **1** and **2** (1 M in THF) by DACH **3** or DIPEDA **4** (10% mol) leads to the formation of warfarin in 78(+)/22(-) or 86(+)/14(-) er enantiomeric ratio and yields >90% after 24 or 48 h respectively [22].

Based on this, in order to find the optimal conditions for the ESI experiments, we first test the reaction at lower concentrations using DACH

as catalyst in THF and in ACN at room temperature (r.t.) for 5 h, obtaining the results reported in Table 1 (entries 1-4).

In addition to room temperature, the effect of higher temperatures was also evaluated, taking into account the availability of heating of the ESI source. Subsequently, reactions at 60 °C and at reflux led, as expected, to a decrease in the enantiomeric ratio (Table 1, entries 5-6) and to an increase in yield from 46% to 63%.

Table 1. Optimization of reaction conditions in bulk (a). All the reactions were performed in a round bottom flask under magnetic stirring. (b)Concentration 10^{-2} M: 0.12 mmol of 1, 0.15 mmol of 2 and 10% (0.01 mmol) of catalyst (3 or 4) in 10 mL of solvent. Concentration 10^{-4} M: 0.1 mL of mixture 10^{-2} M are diluted with 9.9 mL of solvent. (c)The er was calculated by enantioselective HPLC analysis (see ESI for more details). (d)The er was determined on reaction crude. (e)The er was determined on the isolated product (see ESI for more details): entry 3: yield 46%; entry 6: 63%; entry 7: yield 49%; entry 8: 68%.

Entry	Bulk reactions ^a					
	Solvent	T (°C)	Conc. ^b	Catalyst	Time	er (+)/(-) ^c
1	THF	r.t.	10^{-2} M	S,S-3	5 h	20/80 ^d
2	THF	r.t.	10^{-4} M	S,S-3	5 h	24/76 ^d
3	ACN	r.t.	10^{-2} M	S,S-3	5 h	15/85 ^e
4	ACN	r.t.	10^{-4} M	S,S-3	5 h	18/82 ^d
5	ACN	60	10^{-2} M	S,S-3	5 h	20/80 ^d
6	ACN	82	10^{-2} M	S,S-3	5 h	33/67 ^e
7	ACN	r.t.	10^{-2} M	R,R-4	5 h	80/20 ^e
8	ACN	82	10^{-2} M	R,R-4	5 h	64/36 ^e

We chose the concentration of 10^{-2} M in ACN to test the reaction with DIPEDA, which gave the results reported in Table 1, entries 7-8. Starting from 19.6 mg of 1, after 5 h of reaction time (entries 3 and 7) correspond to 17 mg and 18 mg of enantioenriched product, respectively, making them

suitable at a preparative level. Having selected the appropriate solvent and concentration, we then carried out the reaction in microdroplets by injecting the 10^{-2} M ACN solutions into a suitably modified ESI Z-spray source.

The ESI microdroplets were collected in an Eppendorf tip containing a 'silica cap' to capture the neutral products of the reaction for their HPLC quantification.

The apparatus used for the microdroplet reaction is shown in Figure 1 and described below.

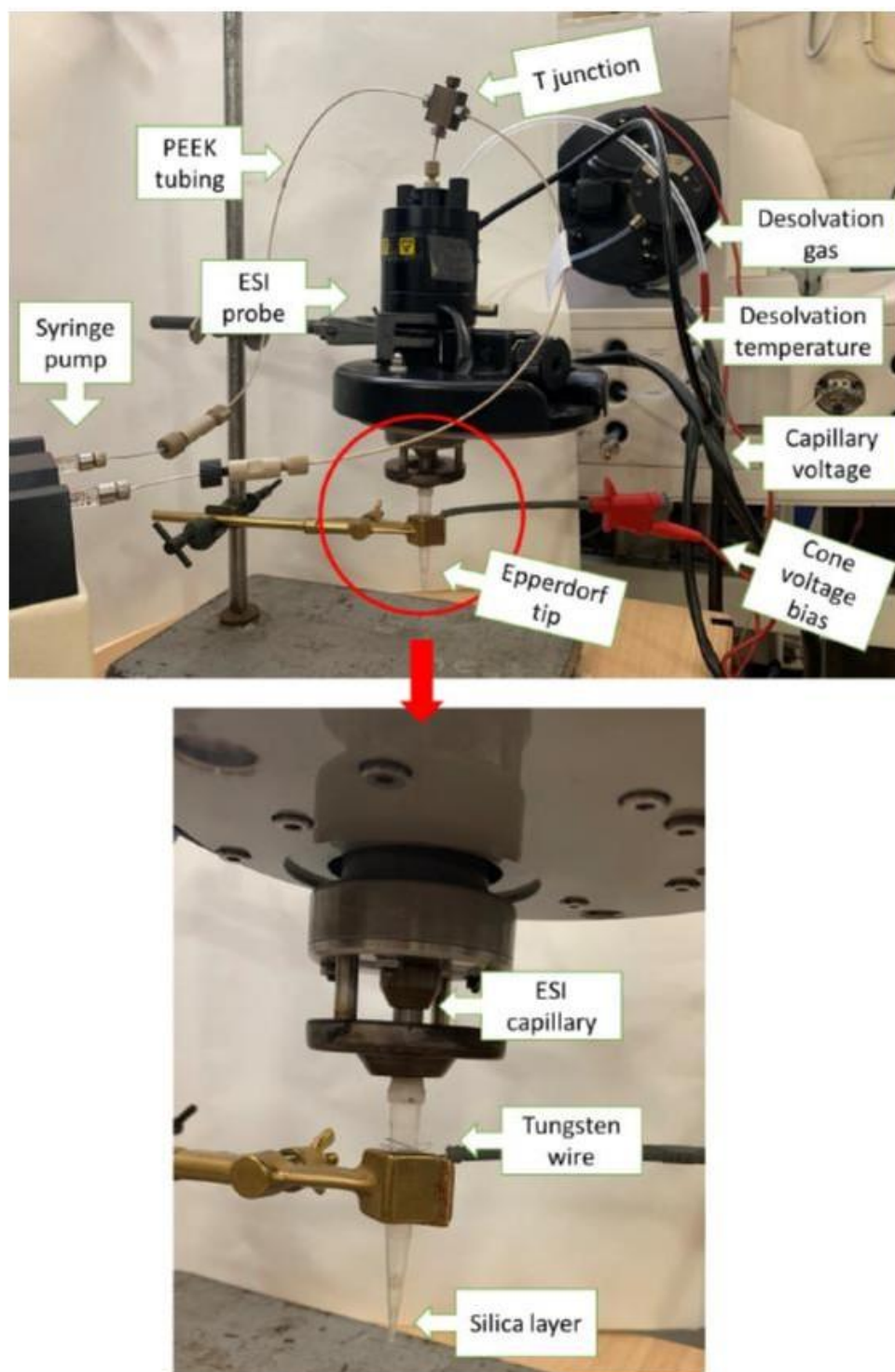


Figure 1. Photograph of the apparatus employed for the Warfarin synthesis in the microdroplet environment.

In the system A, the reactants and catalyst were mixed and infused via a syringe pump in the ESI silica capillary of the source probe through a PEEK tubing (ID = 0.010"). In the system C and system D, a stainless-steel T junction was interposed between the syringe pump device and the ESI probe. As a result, different solutions were separately injected through two PEEK tubs directly connected to the T junction (see following Figures).

Lastly, in system B, the single capillary mounted inside the ESI probe (ID = 0.150 mm) was replaced by two parallel silica capillaries connected to different syringes through two PEEK tubes (ID = 0.010").

The microdroplets generated through the three above-described systems were collected on a 1 cm thick layer of silica placed inside a 1 ml capacity Eppendorf tip. According to this experimental setup, the distance between the spray needle and the silica layer is estimated to be approximately 5 cm. A tungsten wire biased with the ESI source cone voltage (60 V) was inserted in the Eppendorf tip at ca 1 cm from the ESI capillary needle to ensure the high voltage field for droplets coulombic explosion.

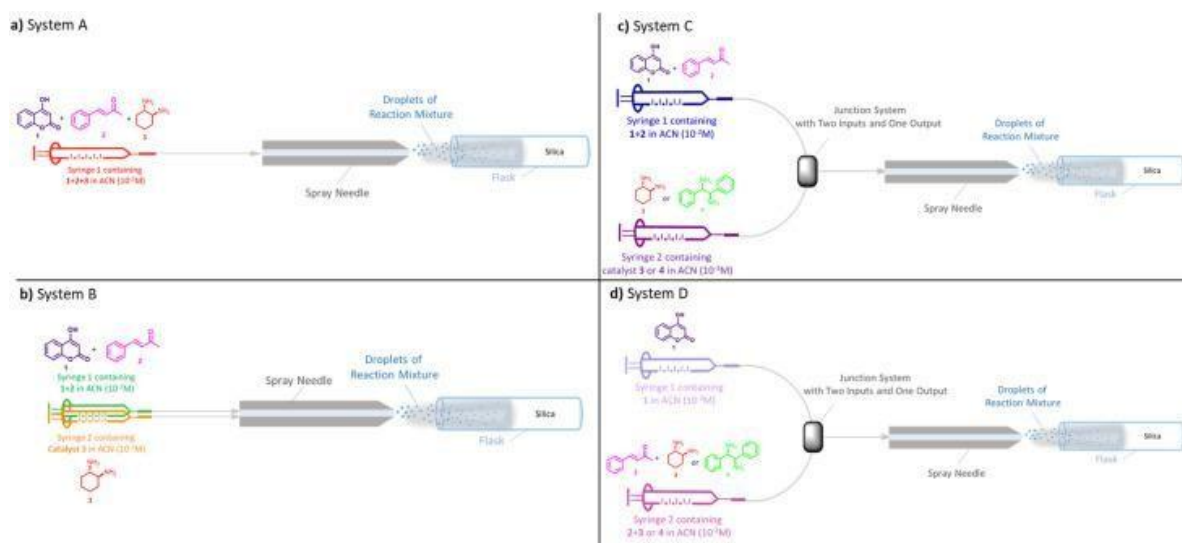


Figure 2. Simplified schemes of microdroplet reaction systems.

Initially, we tested the microdroplet reaction using the System A set-up, which involved a syringe containing the reaction mixture of 1, 2 and catalyst 3 (10^{-2} M solution in ACN) in a single spray (Figure 2a).

The reaction mixture was filled in the syringe and infused at a flow rate of $15\ \mu\text{L min}^{-1}$ for 5 hours in order to collect a manageable quantitative of crude.

The crude of reaction was collected and analyzed by enantioselective HPLC. The value of found e.r. (entry 1, Table 2) was lower than that obtained from the reaction in bulk at room temperature but higher than the bulk reaction performed at reflux temperature, despite the measured temperature of ESI source is $85\ ^\circ\text{C}$ (desolvation gas temperature (DSG) setting at $150\ ^\circ\text{C}$).

Although the set-up of System A involving the reaction mixture with all the reactants placed in a single syringe is widely used for microdroplet reactions, [2a,5a,19] we considered the possibility to make an error due to a not negligible mixing time of the reagents within the syringe. To avoid this, we employed the System B set-up (Figure 2b).

In the System B the solution of reagents is flushed separately from that of the catalyst in two capillary liners inserted into the same ESI spray needle.

Unfortunately, higher quantities of unreacted 4-hydroxycoumarin, and lower *er* (Table 2, entry 2) were recorded.

Table 2. Microdroplet reactions conditions.

General: all the reactions in microdroplet were performed using a modified Z-spray source. (b) Measured temperatures of the microdroplets: when DSG = 150 °C (dSG, desolvation gas temperature), measured T = 85 °C; when DSG = 50 °C, measured T = 25 °C. (c) c.t.: collection time. (d) *er* was calculated by enantioselective HPLC. (e) *er* was determined on reaction crude. (f) Yield and *er* were determined on the isolated product. (g) n.d.: not determined.

Entry	System	Catalyst	T (°C) ^b	Source distance	c.t. ^c	<i>er</i> (+)/(-) ^d	Yield ^f
1	A	S,S-3	85	5 cm	5 h	23/77 ^e	n.d. ^g
2	B	S,S-3	85	5 cm	5 h	38/62 ^e	n.d. ^g
3	C	S,S-3	85	5 cm	5 h	25/75 ^f	10%
4	C	S,S-3	85	8.5 cm	5 h	47/53 ^e	n.d. ^g
5	C	None	85	5 cm	5 h	—	—
6	C (0 kV)	S,S-3	85	5 cm	5 h	31/69	n.d. ^g
7	C	R,R-4	85	5 cm	5 h	74/26 ^f	16%
8	D	S,S-3	85	5 cm	5 h	25/75 ^f	35%
9	D	R,R-4	85	5 cm	5 h	78/22 ^f	23%
10	D	R,R-4	25	5 cm	5 h	76/24 ^f	45%
11	D	R,R-4	85	1 cm	5 h	77/23 ^f	79%
12	D	R,R-4	25	1 cm	5 h	75/25 ^f	71%
13	E	R,R-4	25	1 cm	5 h	75/25 ^f	75%

This arrangement probably leads to inefficient microdroplets mixing.

A good solution is represented by System C, where a “T junction” at zero void volume was introduced as feed of reagents/catalyst. In detail, one syringe contains the solution of 1 and 2 in ACN while the second syringe contains the solution of catalyst 3 or 4 (Figure 2c).

Once collected the reaction crude, a higher formation of warfarin has been highlighted by TLC monitoring, making possible its isolation by chromatographic purification. The chromatogram of isolated warfarin showed an *er* comparable to the bulk reaction at r.t. and higher respect to the *er* obtained in bulk at reflux temperature (Table 2, entry 3).

Taking into account the high temperature involved, we checked the stereostability of warfarin by performing racemization studies on a scalemic mixture of pure warfarin using both bulk and microdroplets conditions.

The enantiomeric excess did not change after 5 h of reaction attesting the stability of the warfarin produced in those conditions.

System C was then tested increasing the distance of the ESI probe from 5 cm to 8.5 cm from the collecting system but lower formation of the product and lower e.r. were observed (Table 2, entry 4). In addition, we tested microdroplet reaction in absence of catalyst and no formation of warfarin was observed (Table 2, entry 5).

The reaction was then performed in absence of the electric voltage applied to the ESI spray needle as proof of the beneficial effects of the ESI source. In this case, the formation of the product occurs with very low yields (traces) and *er* (Table 2, entry 6). Finally, best reaction conditions found using System C (5 cm, 85 °C, positive voltage) were also used with R,R-4 catalyst which confirmed the results obtained with catalyst 3 leading to 74/26 *er* (Table 2, entry 7).

Afterwards, System D (Figure 2d), was tested as clone of System C except for the reagents mixing mode. Here, the solution of 4-hydroxycoumarin 1 is placed in the syringe 1 while the solution of ketone with catalyst (2 + 3/4) is placed in the syringe 2. Before starting the microdroplet reaction, benzylideneacetone and catalyst were left in solution for 30 minutes in order to facilitate the iminium ion formation.

The reaction gave better results in terms of warfarin formation for both the catalysts, even if the yields were still low.

Concerning S, S-3 catalyst, the stereoselectivity remained comparable to that obtained in bulk conditions at r.t. (Table 2, entry 8), while using R, R-4 catalyst the *er* was slightly higher than that obtained using System C but lower than that obtained in bulk conditions at r.t. (Table 2, entry 9). The same reaction performed at room temperature gave higher yields, whereas the *er* remained almost unchanged (Table 2, entry 10).

Further tests were carried out fixing the ESI source at the distance of 1 cm from the collection system.

Unlike what is mostly reported for other microdroplet reactions, [1,2a, b] the use of a shorter distance led to considerably higher yields both at 25 °C

and at 85 °C while no effect on the enantioselectivity was observed (Table 2, entries 11 and 12).

The temperature decrease seems to be negligible at this distance if compared to the results obtained at the distance of 5 cm.

The higher yields observed at the shorter distance can be explained by the formation of a naked-eye visible thin film onto the silica layer by microdroplets deposition. In this condition, the reaction may also proceed in the confined volume of the thin film leading to the increased yields observed.

Noteworthy is that in bulk conditions these values of yields are obtained after about 24 hours while in microdroplet/thin film conditions were obtained in only 5 hours.

Finally, taking into account that the arrangement where the sprays intersect is highly desirable, [21] the last tested system involves two different ESI sources located at an angle of 60° and at 1 cm of distance from the silica cap (System E, Figure 3)

This assay led to the formation of the product with a 75% yield and 75/25 *er* (Table 2, entry 13). Results are comparable with those obtained with System D in entries 11 and 12. Overall, the use of System D at the distance of 1 cm from the collection system and at the temperature of 85 °C turned out to be the best solution both for the yields, the stereoselectivity, and the greater handless and ease of the experimental conditions which involve only one ESI spray probe.

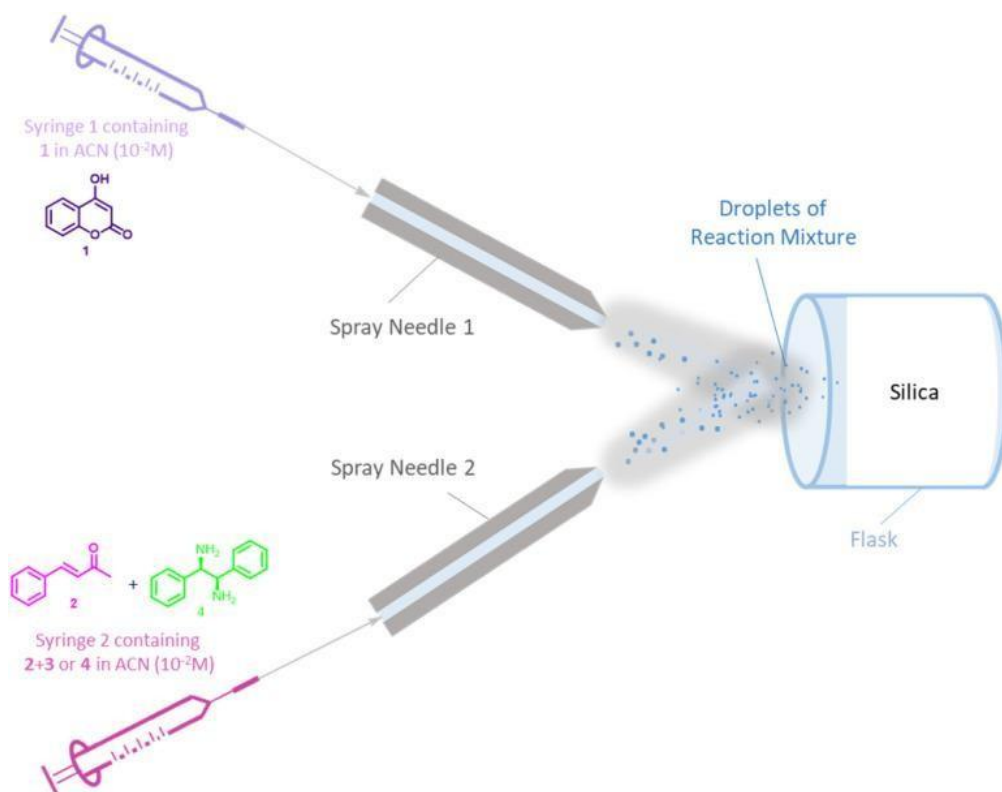


Figure 3. Simplified scheme of system E. The two spray needles are located at an angle of 60°, at the distance of 1 cm from the silica cap.

To probe that the reactions occur in microdroplets environment, on-line reaction monitoring was performed by acquisition of the mass spectra of the solutions containing the DACH catalyst sprayed in the ESI source by using the same A, C and D systems. When the reaction with DACH is performed using System A, the mass spectrum shows the signal of the imine intermediate at m/z 243 and of the warfarin final product at m/z 309 (Figure 4).

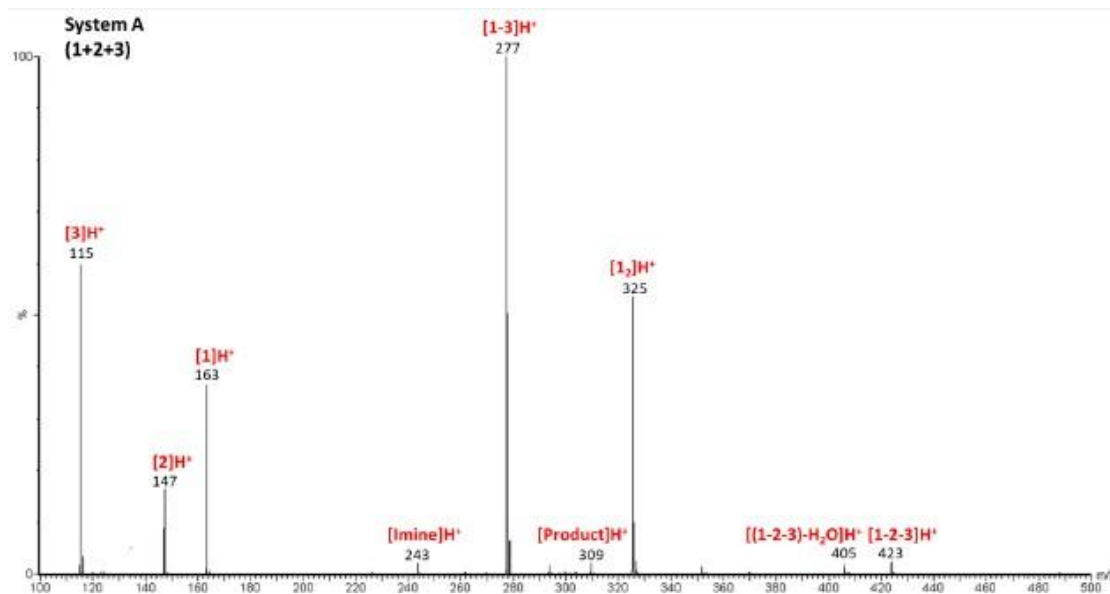


Figure 4. Figure displays the positive electrospray mass spectrum (ESI-MS) recorded before the infusion of the (1+2+3) solution through the System A.

Major ionic peaks are assigned to the protonated catalyst 3 ($m/z = 115$), the protonated reactant 1 ($m/z = 163$) together with the corresponding dimer ($m/z = 325$), the protonated adduct between 1 and 3 compounds ($m/z = 277$), and the protonated reactant 2 ($m/z = 147$).

Minor ion at $m/z = 243$ is attributable to the imine species formed by the reaction of the reactant 2 with the catalyst 3.

This intermediate is notoriously involved in the reaction mechanism leading to the formation of the final product that is detected at a low intensity as the protonated species at $m/z = 309$. Also, in this case, it is not possible to exclude the contribution of the solution reaction in the formation of the final product. To evaluate the effective contribution of the microdroplet environment in generating the reaction intermediate and then the final product, a mixture containing both 1 and 2 (1+2) was infused separately from a solution of the catalyst 3 through the System C (Figure 4).

However, the formation of the imine intermediate and warfarin even in the syringe during the time required to spray 1 mL of the freshly prepared solutions (50 minutes) may not be negligible.

Nevertheless, when the reaction is performed using System C, where the reactants and the catalyst are separated, this contribution is avoided. In

spite of the low ion intensities, the fact that signals of the imine intermediate and of the product are measured under these conditions (Figure 5) demonstrates the formation of warfarin by microdroplets reaction.

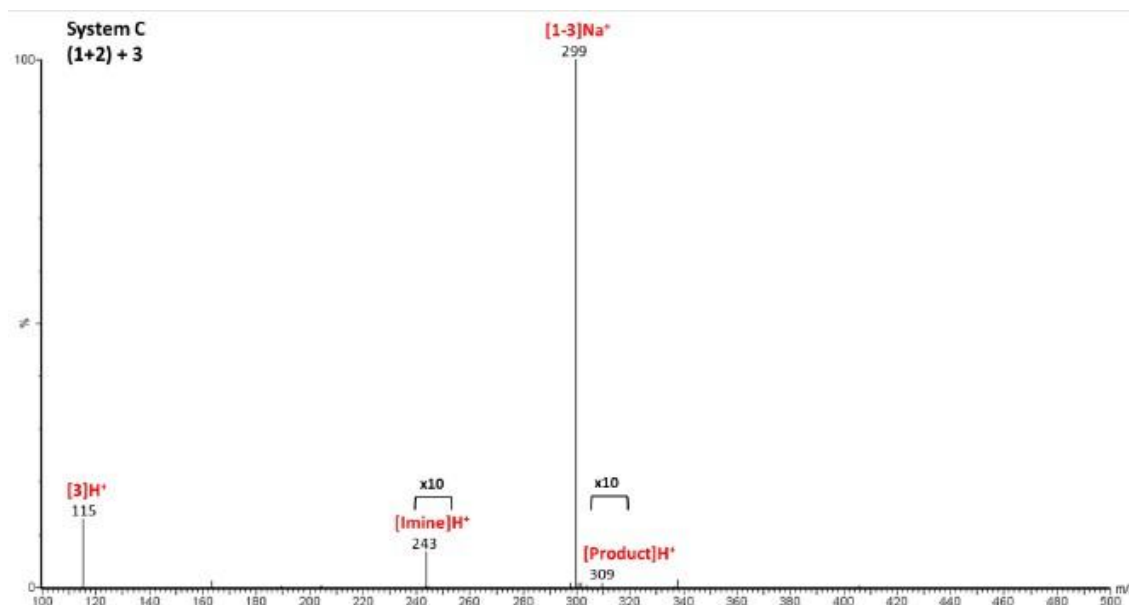


Figure 5. ESI-(+) mass spectrum of the (1+2) and 3 solutions submitted to reaction through the System C.

In this case, the MS can be considered representative exclusively of the reaction occurring in the microdroplet environment since the two reactants (1 and 2) encounter the catalyst only reaching the T junction.

Therefore, the imine intermediate at m/z 243 is necessarily formed in the confined volume of the microdroplets generated during the electrospray process. Little amount of the protonated product at m/z 309 was also detected.

To improve the efficiency of the reaction, a mixture containing both 2 and 3 (2+3) was infused separately from a solution of the reactant 1 through the System D (Figure 8).

When System D is used (Figure 6) the higher imine intermediate ion intensity is justified by its concomitant formation in the syringe thus leading to a slightly higher ionic intensities of the final product. Similar results were obtained with catalyst 4.

Nevertheless, the very high yields observed with System D can be explained by the generation of a thin film within which the reaction can proceed with further products formation.

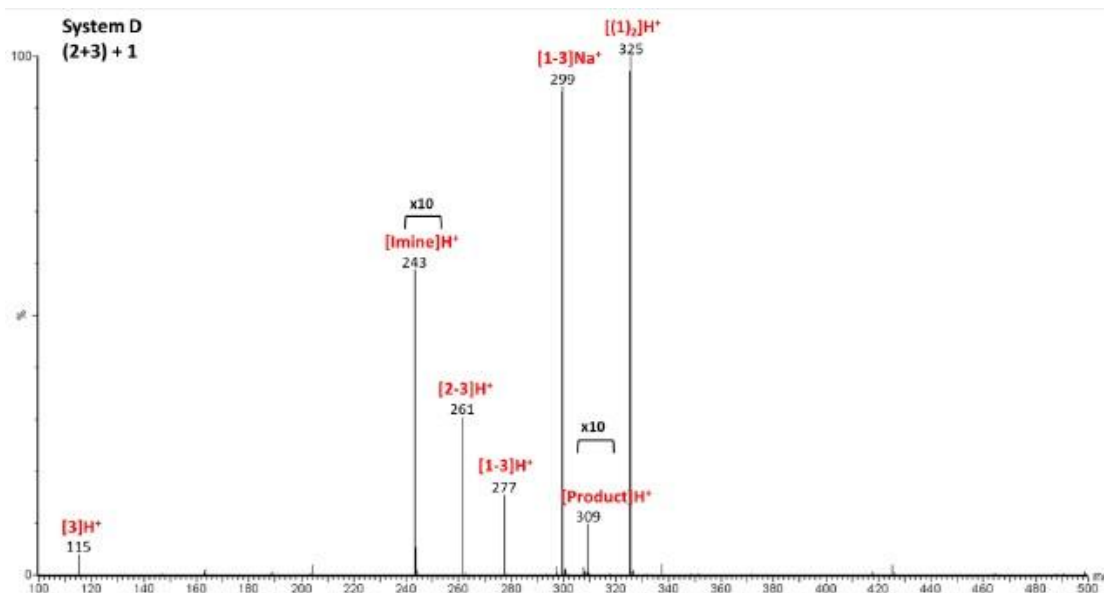


Figure 6. ESI-(+) mass spectrum of the (2+3) and 1 solution submitted to reaction through the System D.

In this case, the imine intermediate at m/z 243 can be obtained not only from an accelerated reaction in the microdroplet environment, but also in solution during the residence time of the (2+3) mixture in the syringe used for the injection.

The increase of the intermediate concentration because of the two parallel processes determines a predictable increase of the product detected at m/z 309.

If the ionic intermediate can be formed from different pathways (solution + microdroplets), the final product only arises from a microdroplet reaction since the 4-hydroxycoumarine 1 encounters the imine intermediate only at the level of the T junction.

6.3 Conclusion

Concerning the warfarin synthesis, the achieved results about stereoselectivity are comparable to those obtained in bulk conditions at room temperature, and at the same time increase if compared to the bulk reaction performed at higher temperature.

Notably, the use of the positive voltage of the ESI source enhances the yields and the stereoselectivity of the reaction underlying the role of the charged microdroplets to accelerate the reaction as also evidenced by the on-line MS reaction monitoring experiments.

Yields up to 79% and 77/23 *er* were obtained when the reaction time was extended by the formation of a thin film from the microdroplets deposition at shorter distance from the silica layer giving the product in quantities comparable to those obtained in bulk after 24 hours of reaction, in only 5 hours of collection.

Notwithstanding this, our work represents the first example of an asymmetric organocatalyzed reaction in ESI-microdroplets/thin film confined volumes where the stereoselectivity is retained or higher compared to that in bulk conditions, enabling the possibility to merge the advantages of confined volumes reactions with those of the asymmetric organocatalysis.

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7. Charged Microdroplets Deposition for Nanostructured-Based Electrode Surface Modification

7.1 Introduction

Synthesising gold nanostructures has attracted considerable attention owing to their many relevant applications. They are composed of one or more inorganic species, such as noble and heavy metals, with diameters ranging from 1 to 100 nm [1]. Their high surface-to-volume ratio, surface energy and conductivity, as well as their biocompatibility and ease of functionalisation, make them fundamental in the development of biosensors. Gold nanoparticles (AuNPs) not only increase the electrode area, but also show a remarkable affinity for proteins, promoting greater protein surface coverage to preserve their biological activity [1-3].

Gold nanoparticles can be conjugated to enzymes by electrostatic or covalent methods, promoting a favourable orientation of the biomolecules and electron conduction between the prosthetic group of the enzyme and the electrode surface. In addition, this procedure often prevents possible conformational changes of the enzyme due to direct absorption at the electrode.

This promotes a more intense and selective interaction with the gold nanoparticles, thus improving the electron transfer [4,5]. One of the challenges in using AuNPs for various applications is to control their size, shape and surface chemistry.

Controlling the size and shape of nanostructures is important because both factors strongly influence the optical, mechanical, electrical and chemical properties of the nanostructures themselves.

Gold nanoparticles can be prepared in solution by reduction of chloroauric acid (HAuCl_4) in the presence of reducing agents such as sodium borohydride, citrates or ascorbates.

After dissolving the chloroauric acid (HAuCl_4) in water, the solution is stirred and the reducing agent is added.

This causes the reduction of Au^{3+} ions to neutral gold atoms.

As the concentration of these atoms increases, the solution becomes saturated and the gold gradually precipitates into sub-nanometric particles. The gold atoms that continue to form attach themselves to the already precipitated particles; if the solution is stirred vigorously enough, the particles will be of a fairly uniform size.

Zare and colleagues recently demonstrated that AuNP can be formed by ESI microdroplet deposition under ambient conditions by spraying a solution containing only tetrachloroauric acid without the addition of reducing agents [6].

In addition, AuNP less than 10 nm in diameter have been produced on the microsecond time scale.

The rapid formation of AuNP was attributed to the strong electric field at the water-air microdroplet interface.

By exploiting the properties of aqueous microdroplets, they achieved the formation of AuNPs even in the absence of any added reducing agent or externally applied charges through a process that is accelerated by five orders of magnitude compared to the same reaction in bulk.

In this study, the process developed by Zare et al. was used to modify the surface of graphite screen-printed electrodes (GSPE).

The morphology of AuNP was characterised by scanning electron microscopy (SEM), while cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) were used to study the electrochemical behaviour of the modified electrodes. In addition, the same deposition method was used to functionalise the AuNP-modified electrode surface with 6-ferrocenyl hexanethiol (6Fc-ht) as a redox probe.

Functionalisation of AuNP with organic molecules such as thiols can alter their surface properties and improve their stability, biocompatibility and functionality.

7.2 ESI Microdroplets Deposition Experiments

To investigate gold formation in the Q-TOF ESI Z-spray modified source, a stream of aqueous microdroplets from a 6.4×10^{-4} M HAuCl_4 solution was collected on a glass slide in negative ion mode for 1 h.

The formation of a gold film visible to the naked eye clearly demonstrated the ability of our systems to reproduce the Zare experiments (Figure 1).

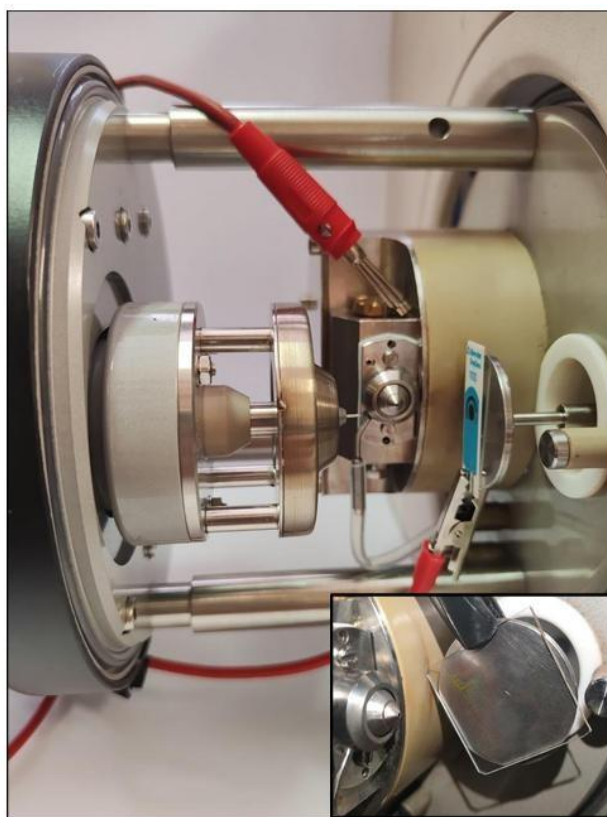


Figure 1. ESI Z-spray source apparatus for charged microdroplets deposition onto screen-printed electrodes. Inset: Gold particles deposited onto a glass slide after 1 hour of 6.4×10^{-4} M HAuCl_4 solution deposition.

Moreover, even the negative ion mass spectrum registered by the Q-TOF mass spectrometer was comparable to that reported by Zare et al., showing the signal corresponding to AuCl^{2-} and AuCl^{4-} as the predominant anionic

species produced by the complete desolvation of the ESI microdroplets Figure 2.

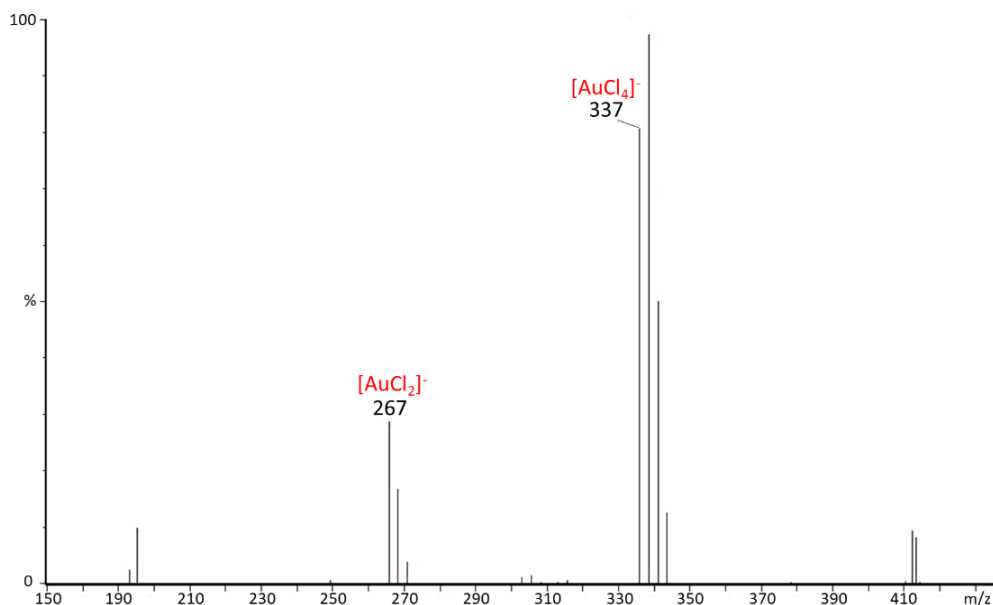


Figure 2. ESI negative mass spectrum of $6.4 \times 10^{-5} \text{ M HAuCl}_4$ water solution.

As pointed out by Zare, the AuCl_2^- species clearly indicates that Au^{+3} is reduced inside the microdroplets. In order to promote the deposition of gold nanoparticles with nanometric diameters, the tetrachloroauric acid solution was diluted tenfold ($6.4 \times 10^{-5} \text{ M}$) and the reaction time was reduced in the range of 2-10 min. The chosen deposition times are considerably longer than the millisecond interval used by Zare et al. in their experiments. This choice was necessary to obtain intense electrochemical signals, but led to an increase in the size of the gold nanoparticles, which underwent aggregation phenomena (vide infra).

The ESI microdroplets were collected on the working surface of a GSPE.

ESI source parameters, such as electric field polarity and spray-target distance, can strongly influence the microdroplet deposition processes [7,8].

In a series of preliminary experiments, the distance between the ESI spray tip and the specific part of the GSPE to be modified by AuNPs was varied from 1.0 to 3.5 cm.

DPV analysis of the AuNPs-modified electrodes indicated that the optimum electrochemical signal was associated with the deposition of the ESI microdroplet stream at a distance from the graphite electrode of 2.5 cm.

This parameter was then used to modify the working area of the AuNPs modified GSPE electrodes in all subsequent experiments.

Three experiments were performed to determine the efficiency of AuNP deposition by extending the deposition time to 150 min to achieve weighable gold on the GSPE surface.

The modified electrodes were washed with water to remove chloroauric acid residues and placed in an oven at 100 °C for 1 h.

The measured increase in electrode weight corresponds to an average conversion of 60% of the total chloroauric acid delivered to the surface.

Microdroplets containing 6Fc-ht as a redox mediator were then deposited on the AuNPs-modified electrodes.

The only difference in the mass spectrometric conditions used in these experiments was the ESI voltage polarity, which was switched to positive values, leading to better results in terms of DPV and CV measurements. The positive ESI mass spectrum of 6Fc-ht is dominated by a single signal corresponding to its molecular ion at m/z 302 (Figure 3).

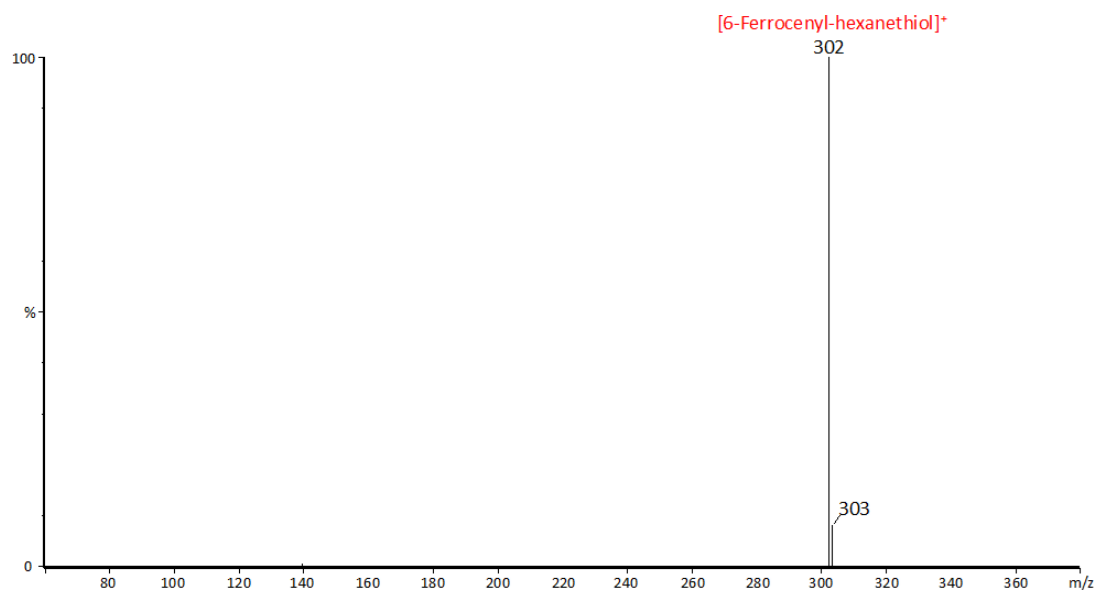


Figure 3: ESI positive mass spectrum of 1×10^{-5} M 6-Ferrocenyl-hexanethiol methanol solution.

7.3 SEM and Electrochemical Characterization of the AuNPs-Modified Electrodes

The morphology and distribution of the AuNPs obtained through the deposition of ESI microdroplets after 2, 4 and 10 minutes of a HAuCl_4 solution were analysed under an SEM microscope (Figure 4).

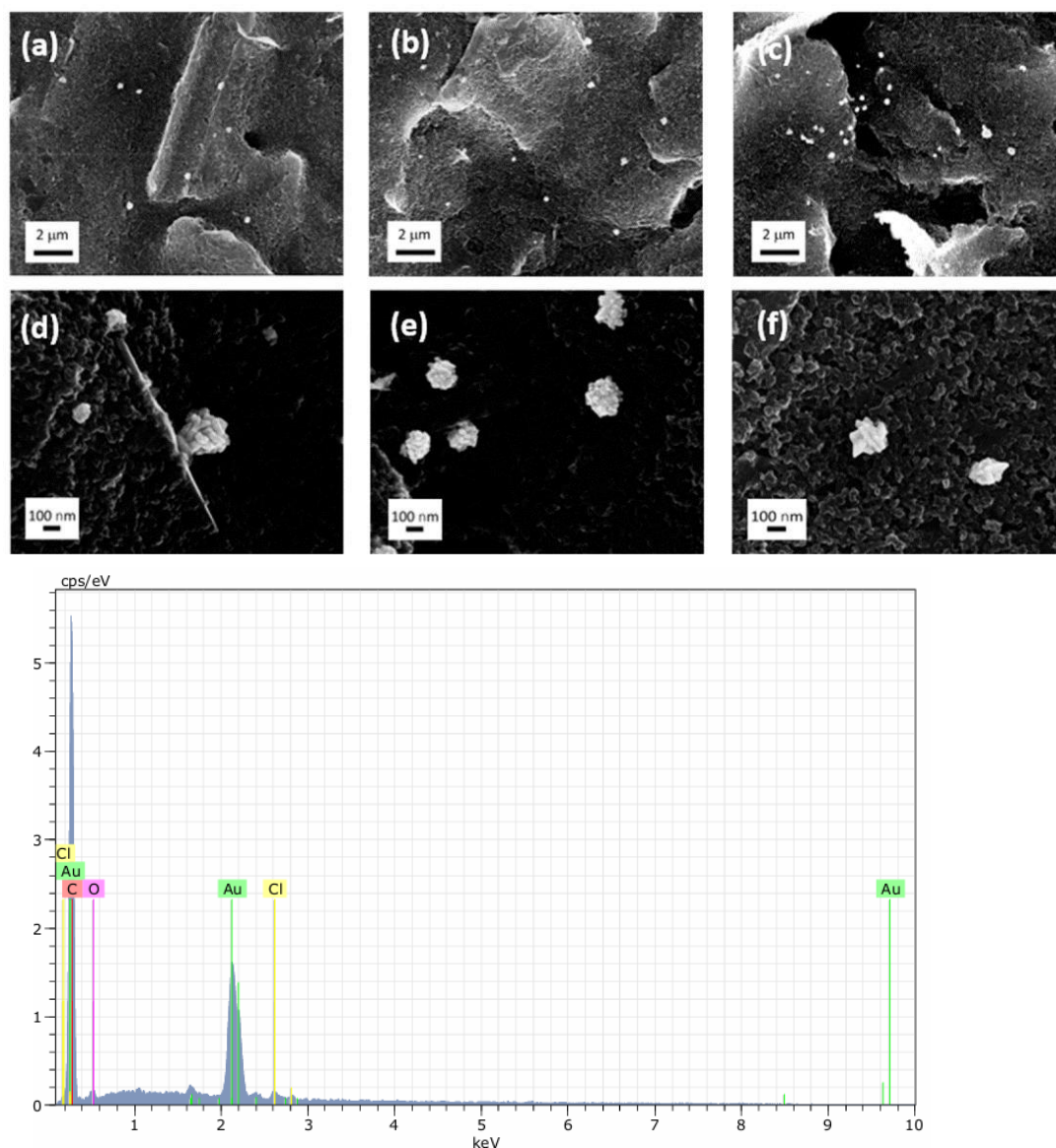


Figure 4. SEM images: (a, d) 2 min of deposition; (b, e) 4 min of deposition; (c, f) 10 min of deposition. Below, EDX analysis of the graphite screen-printed electrode after 10 minutes of microdroplets deposition of HAuCl_4 solution.

The total amount of HAuCl_4 delivered to the electrode surface also increases by increasing the deposition time: 2 min: 0.00087 mg; 4 min: 0.0017 mg; 10 min: 0.0043 mg.

Between 2 and 4 min, the number of AuNPs increases accordingly and the distribution over the surface appears homogeneous after 4 min.

After 10 min of deposition, the AuNPs start to concentrate more in specific regions (Figure 4a-c). The AuNPs morphology results in large anisotropic aggregates of smaller NPs (of about 30 nm), which are not completely formed after 2 min of deposition (Figure 4d and Figure 5), with the co-presence of AuNPs of about 350 nm and 100 nm.

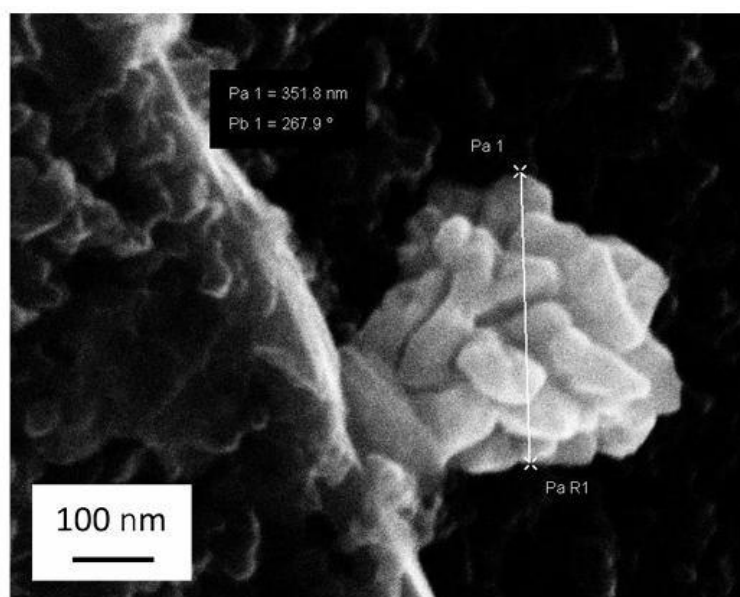


Figure 5. SEM image reporting the enlargement of AuNPs aggregates obtained with 2 minutes of deposition.

After 4 min (Figure 4e), the medium size for all the AuNPs aggregates is approximately 220 nm, and the aggregates are formed by smoother NPs (Figure 6). After 10 min of deposition, the medium size is approximately 260 nm with more spikey morphology (Figure 4c, f and Figure 6). In addition, the presence of random bigger aggregates suggests the occurrence of successive nucleations during the longer deposition time.

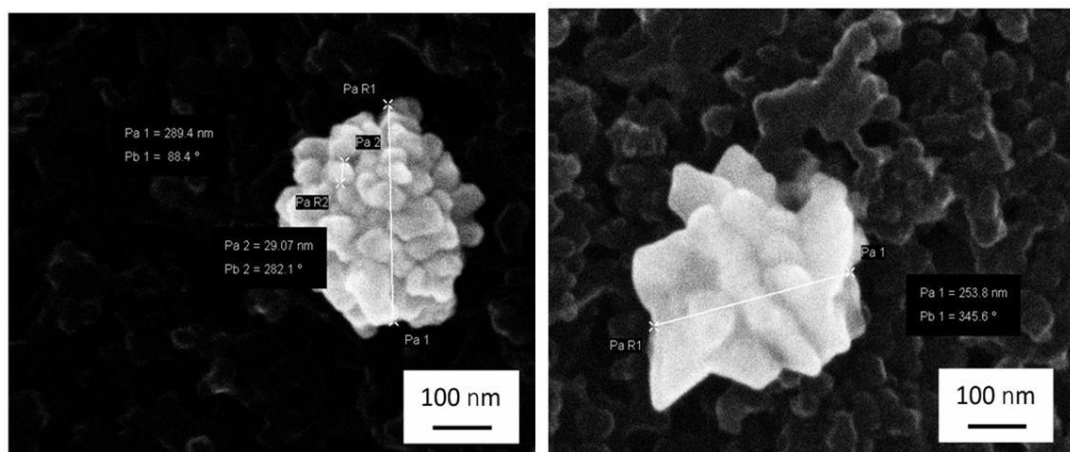


Figure 6. Left, SEM image reporting the enlargement of AuNPs aggregates obtained with 4 minutes of deposition. Right, SEM image reporting the enlargement of AuNPs aggregates obtained with 10 minutes of deposition.

The graphics of the statistical distribution of AuNPs content and size for three different batches at 2, 4, and 10 min of deposition are reported in Figures 7–9.

An increase in the average diameter of the deposited AuNPS can be observed going from 2 to 10 minutes of deposition.

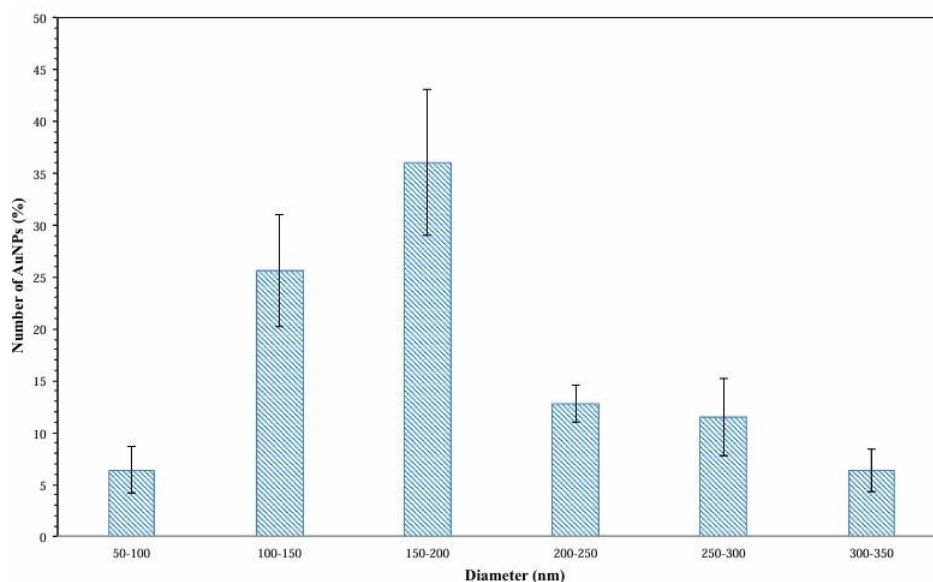


Figure 7. Size distribution of AuNPs after 2 minutes of microdroplets deposition.

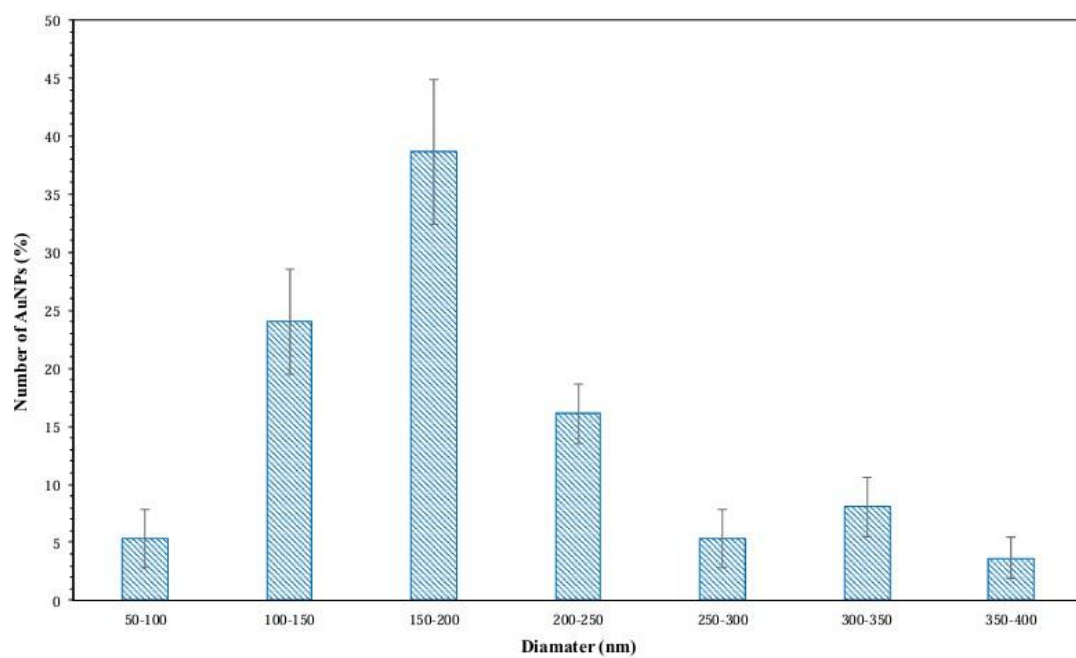


Figure 8. Size distribution of AuNPs after 4 minutes of microdroplets deposition.

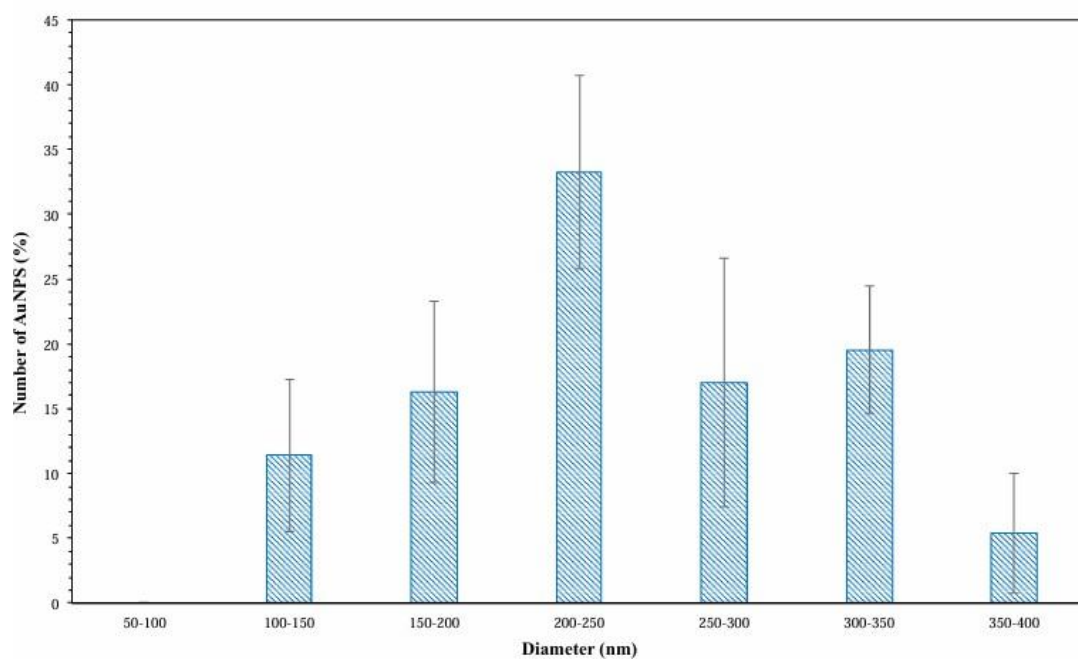


Figure 9. Size distribution of AuNPs after 10 minutes of microdroplets deposition.

The electrochemical characterization of GSPE/AuNPs platforms was realized using CV, DPV, and EIS measurements.

The CV curves (Figure 10) show increasing oxidation and reduction peak currents for the redox couple $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ with increasing HAuCl₄ deposition time, as well as the reversibility of the reaction. These results are consistent with the widely known property of AuNPs to promote ET efficiency, increasing the surface-to-volume ratio, as well as the electrode conductivity [9-14].

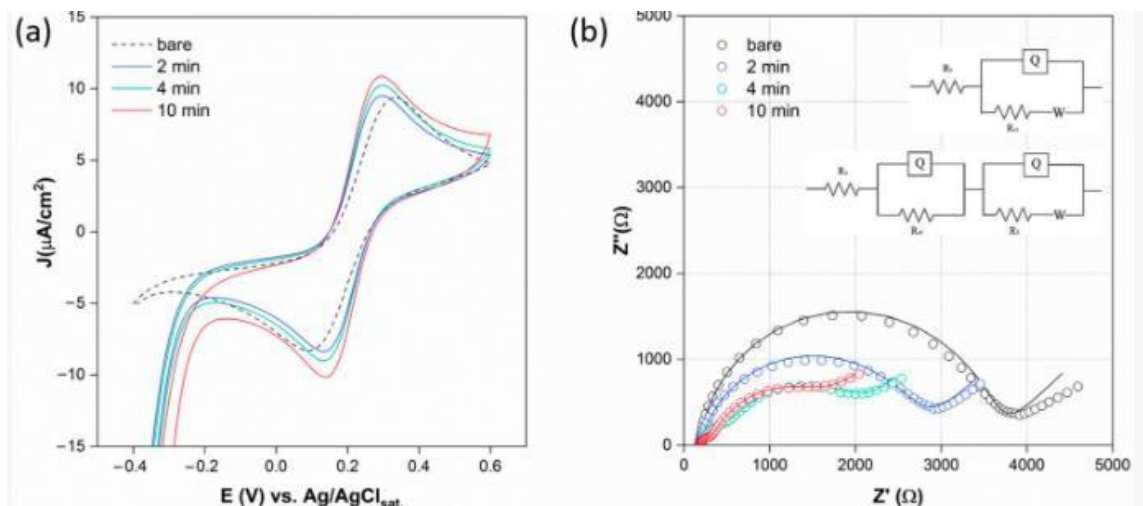


Figure 10. (a) CV measurements of AuNPs-GSPE surfaces modified under different deposition times, performed in $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 1 mM, KCl 0.1 M, between -0.4 and 0.6 V vs. Ag/AgCl_{sat.}, rate = 10 mV/s. (b) EIS measurements of AuNPs-GSPE surfaces under different deposition times, performed in $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 5 mM, KCl 0.1 M, EDC = 0.215 V, EAC = 0.01 V, v_{max} = 100,000 Hz, v_{min} = 0.1 Hz. The dotted lines represent experimental data, and the solid lines represent the fit lines. Inset: Randles ($R(Q[RW])$) and $R(QR)(Q[RW])$ circuits employed for the fitting procedure.

The EIS measurements are made for the same platforms and the Nyquist plots are shown in Figure 10b. The impedance curves at 0 and 2 min are fitted with a typical Randles circuit, while those at 10 min are fitted with an $R(QR)(Q[RW])$ circuit, obtaining the characteristic system parameters reported in the supplementary material (Table 1).

Table 1. Parameters obtained by EIS fitting.

Deposition time	$R_{CT} (\Omega)$	$R_{AuI} (\Omega)$	$W (\sigma)$
0	3423	/	648
2	2515	/	583
4	1258	479	563
10	1246	186	516

All platforms are semi-reversible systems, according to the relationship $R_{CT} \geq RW = \sigma/\Omega$, which is the condition for having a visible kinetic region over the mass transfer region [15].

As the deposition time increases, the RCT decreases and, interestingly, at 4 and 10 min, an additional small semicircle appears at higher frequencies (ν). This resistance and capacitance are due to the AuNPs interface (AuI), which becomes visible at higher AuNPs concentrations [16].

However, the presence of AuNPs not only increases the electrode conductivity but also imparts intrinsic heterogeneity to the electrode surface, which is responsible for non-uniform bilayer formation. Therefore, the capacitance contribution decreases as well as both τ_{CT} and τ_d . The Bode plots in Figure 12a,b show the variation of $|Z|$ and phase angle (ϕ), respectively, with increasing applied ν . The plateau region under high ν in the $|Z|$ profile results in ν -independence for all modified electrodes, following the typical resistive behaviour under such conditions [17].

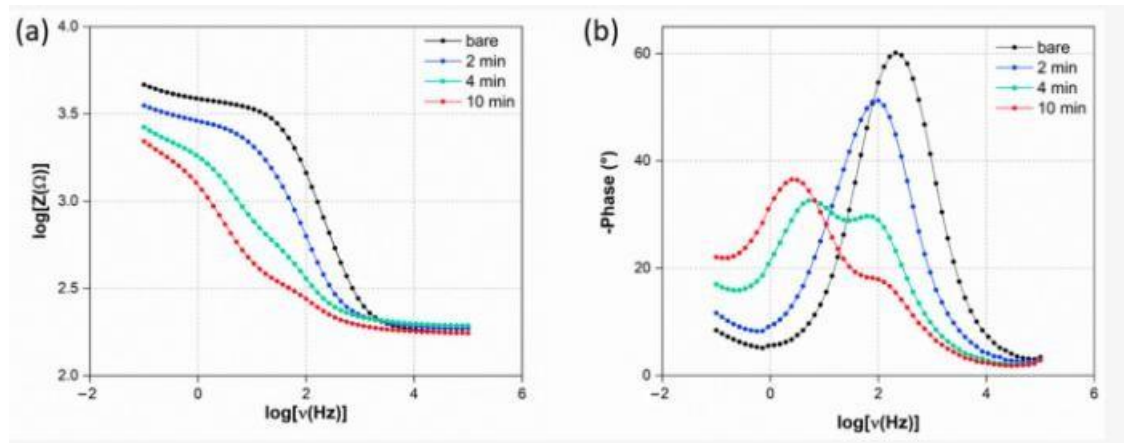


Figure 11. Bode plots (a) $|Z|$ and (b) phase angle behavior in the function of applied ν .

The slope depends on the capacitance of the system, which is responsible for the increasing phase shift between the current signal and the applied voltage. The greater the phase shift, the poorer the current flow through the system. After 2 min of deposition, $|Z|$ decreases relative to the bare electrode over the entire range of applied ν , and $\phi_{\max}$ decreases, shifting slightly to lower ν .

This indicates that the system still exhibits capacitive dominance. With increasing deposition times of 4 and 10 min, both $|Z|$ and ϕ profiles decrease in magnitude compared to the bare and 2 min electrodes and show a splitting into two distinct phenomena, reflecting the behaviour of the Nyquist plot.

The phenomenon at intermediate ν represents the AuI contribution, while the one at lower ν is related to the double layer. After 10 min, despite the decrease in the magnitude of $|Z|$, the $\phi_{\max}$ related to the double layer capacitance increases and shifts towards lower ν .

This is likely due to the formation of a more uniform double layer as a result of reaching a concentration of AuNPs that promotes an increased double layer capacitance compared to the 4 min electrode. In addition, the increasing slope of the $|Z|$ profile at low ν is notable, which can be attributed to the progressive decrease in τ_{CT} , making diffusion the rate-limiting step of the process [18].

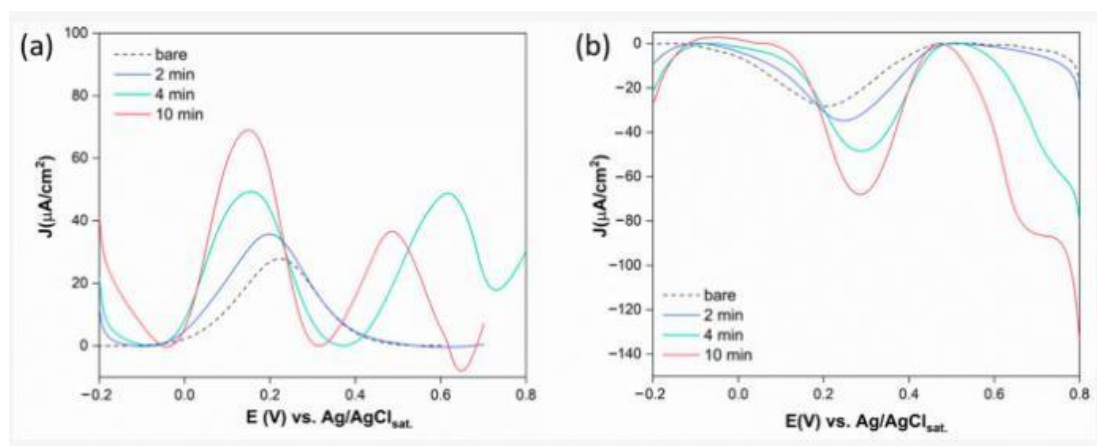


Figure 12. DPV measurements of AuNPs-GSPE surfaces under different deposition times, performed in $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ 1 mM, KCl 0.1 M, EDC = 0.215 V, EAC = 0.01 V, $\nu_{\max} = 100,000$ Hz, $\nu_{\min} = 0.1$ Hz.

DPV measurements performed under oxidation and reduction conditions (Figure 12a, b) allowed a better characterisation of the electrochemical behaviour of the modified platforms. For ferrocyanide oxidation, the peak occurs at 0.22 V for the bare electrode and progressively shifts to lower potentials with increasing deposition time (0.2 V at 2 min, 0.15 V at 4 and 10 min).

This indicates a progressively more favourable ET process, favoured by the increasing concentration of AuNPs. The peak current also increases, reaching its highest value at 10 min.

A similar behaviour is observed under reduction conditions. After 4 and 10 min, an additional ET process is observed under both oxidation and reduction conditions, even in the absence of oxygen and ferricyanide, probably due to the oxidation and reduction of Au.

Literature shows that oxidation under acidic conditions is generally unnoticeable for adsorbed spherical AuNPs [19], whereas reduction is observed in DPV at 0.45V vs. Ag/AgCl_{sat}. The highly anisotropic morphology of AuNPs may account for their particularly high reactivity in this study.

The oxidation peak appears at 0.6 V at 4 min and shifts to 0.49 V at 10 min with increasing current intensity. This is probably due to the spikier morphology [20,21] of the AuNPs obtained after 10 min of deposition (Figure 4e, f) and their higher concentration. In the case of reduction, the additional ET process is less pronounced and the current increases with the higher AuNPs concentration.

7.4 Functionalization of GSPE/AuNPs Platforms with 6-Ferrocenyl-Hexanethiol and Electrochemical Detection of H₂O₂

The GSPE/AuNPs platforms characterised above were functionalised with 6Fc-ht as a redox mediator.

The functionalisation was achieved by conventional self-assembled monolayer (SAM) formation and by ESI microdroplet deposition. In both

cases, prior to characterisation, the electrodes were rinsed with abundant water and ethanol to remove the excess of physically adsorbed 6Fc-ht.

The procedure to provide the formation of a SAM of 6Fc-ht onto the GSPE/AuNPs electrodes was realized by dipping the modified electrodes in a 1 mM thiol solution in ethanol for 24 h.

Afterward, the electrodes were left to dry and rinsed with water and ethanol. CV measurements were also performed for these systems (Figure 13a, b).

The system modified by the SAM procedure did not provide significant oxidation and reduction signals for all HAuCl₄ deposition times. This is probably due to the strong adsorption of the redox probe molecules onto the free graphite via the aliphatic chain.

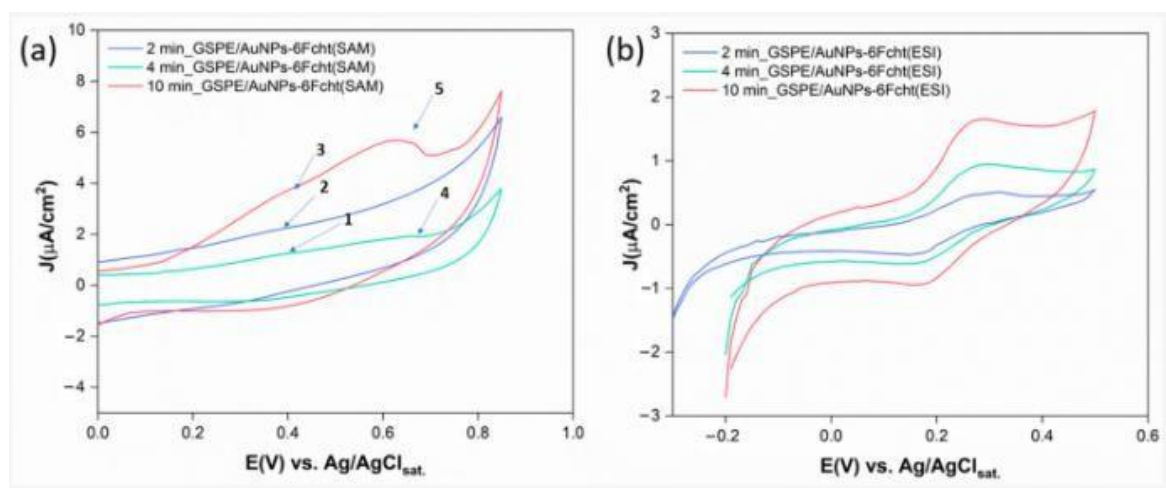


Figure 13. (a) CV measurements of GSPE surfaces functionalized with AuNPs at different HAuCl₄ deposition times, modified with 6Fc-ht through the SAM procedure performed in KCl 0.1 M, between 0 and 0.85 V vs. Ag/AgCl_{sat.}, rate = 10 mV/s. 6Fc-ht oxidation signals (1, 2, and 3) and gold oxidation signals (4, 5); (b) CV measurements of GSPE surfaces functionalized with AuNPs at different HAuCl₄ deposition times, modified with 6Fc-ht through the ESI technique performed in KCl 0.1 M between -0.2 and 0.5 V vs. Ag/AgCl_{sat.}, rate = 10 mV/s.

This induces a high capacitive current and the mobility of the ferrocene is limited. Consequently, only a weak ferrocene oxidation signal is observed in Figure 13a (1, 2, 3) at higher potentials than expected [22].

In Figure 13a (4, 5) at 4 and 10 min, broad gold oxidation signals are also observed.

On the contrary, the ESI deposition technique provided better CV profiles (Figure 13b) even when the electrode surface was exposed to a less concentrated stream of 6Fc-ht microdroplets for only 10 min.

The oxidation and reduction peaks are detected at 0.26 and 0.22 V vs. Ag/AgCl_{sat.} respectively, and the current increases with increasing deposition time.

These results could be attributed to the specific interaction between 6Fc-ht and AuNPs being greatly accelerated compared to the bulk process due to the peculiar properties of the thin film formed by the deposition of charged microdroplets.

Moreover, the limited amount of reagent used to functionalise the gold nanoparticles seems to avoid its physical interaction with the free graphite.

The SAM process, on the other hand, exposes the electrode surface to a concentrated solution of 6Fc-ht over a period of 24 h and is likely to promote its extensive physical adsorption on the free graphite.

Finally, DPV measurements were performed under oxidation and reduction conditions for the systems obtained by different modification procedures (Figure 14).

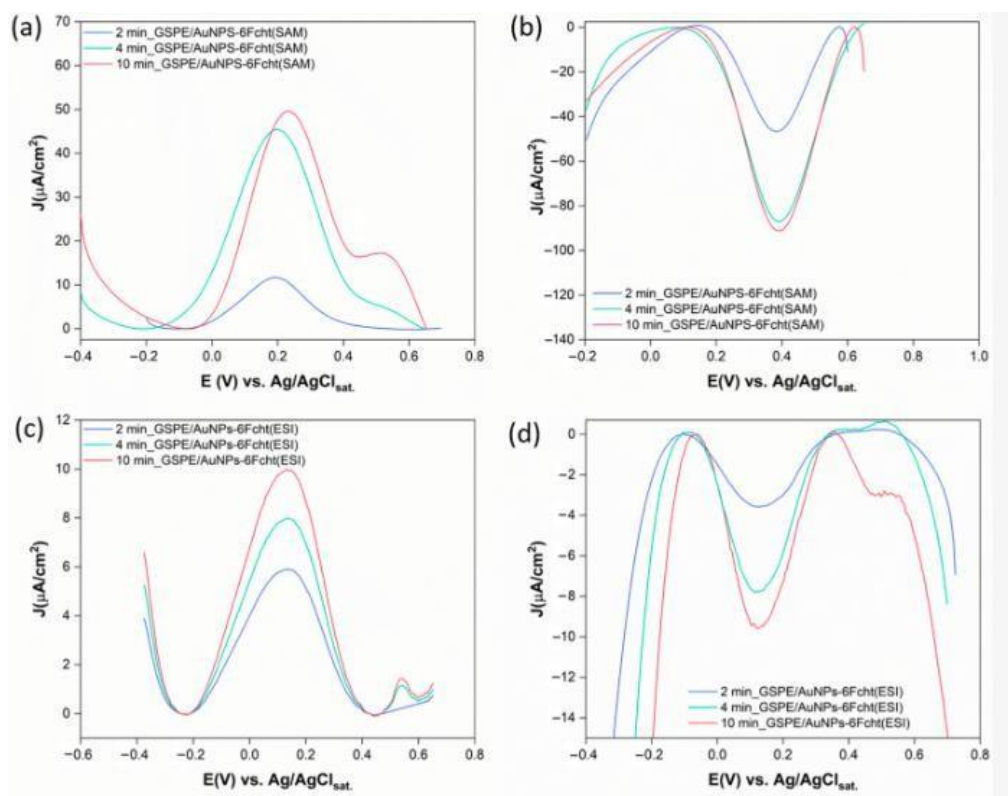


Figure 14. DPV measurements of GSPE surfaces functionalized with AuNPs at different HAuCl_4 deposition times, modified with 6Fc-ht through the SAM procedure (a, b) and ESI microdroplets deposition technique (c, d), performed in KCl 0.1 M, $\text{EDC} = 0.215$ V, $\text{EAC} = 0.01$ V, $v_{\text{max}} = 100,000$ Hz, and $v_{\text{min}} = 0.1$ Hz.

Although oxidation and reduction currents increase with deposition time for the SAM process, the redox process appears irreversible when comparing current intensities.

In addition, the oxidation and reduction potentials are significantly different from those expected for ferrocene.

These results are consistent with the CV data discussed above. In contrast, the DPV for the platforms obtained by the ESI technique shows better reversibility. For all deposition times, oxidation and reduction peaks appear at 0.13 V and 0.12 V vs. $\text{Ag}/\text{AgCl}_{\text{sat}}$, respectively.

This is in agreement with the redox behaviour of ferrocene reported in the literature under similar conditions [23].

Finally, the oxidation of gold is consistently observed after 4 and 10 min of deposition, with the exception of the reduction phase of the SAM system.

For the detection of H_2O_2 , the ESI GSPE/AuNPs-6Fcht platform was used with 10 min of microdroplet deposition, which provides the best electrochemical performance. Specifically, the catalysis of H_2O_2 oxidation by 6Fc-ht was followed by DPV measurements (Figure 15), where the Fc oxidation current increases with increasing H_2O_2 concentration, as recently reported in the literature [24].

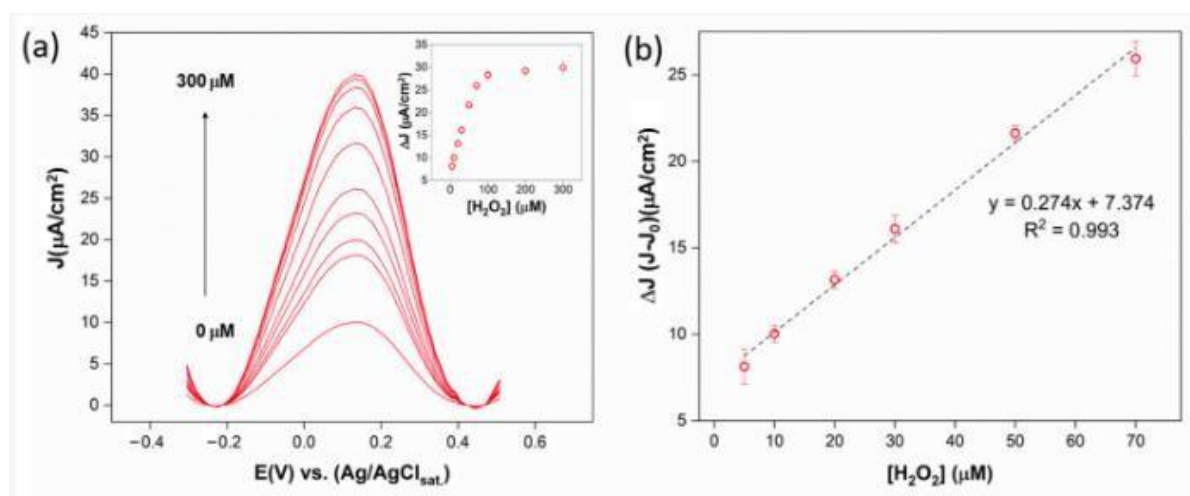


Figure 15. (a) DPV curves collected in the absence and presence of increasing concentrations of H_2O_2 in the range 0–300 μM in PBS 0.01 M, KCl 0.1 M. In the inset are reported the ΔI ($I-I_0$) over the analyzed H_2O_2 concentration range; (b) sensor calibration line.

The mechanism proposed in the literature involves the oxidation of Fc to Fc^+ and the catalysis of H_2O_2 oxidation to O_2 by Fc^+ .

Afterwards, Fc is oxidized back to Fc^+ , increasing the peak current proportionally to the H_2O_2 concentration present in the working solution.

The platform exhibited an optimal response to the target, providing for a linear range of 5–70 μM with a limit of detection (LOD) of 0.9 μM .

These results make this platform promising for applications in reactive oxygen species (ROS) detection.

7.5 Conclusion

This study demonstrates the potential of the ESI-charged microdroplet deposition technique for the formation and functionalisation of AuNPs on GSPEs. By optimising deposition parameters such as ESI voltage polarity and spray-target distance, we achieved a homogeneous distribution of AuNPs, which was verified by SEM analyses.

The advantages of the ESI microdroplet deposition technique over conventional AuNPs generation methods can be summarised as follows:

- low reagent consumption by using HAuCl_4 diluted solution in the absence of reducing agents;
- accelerated nanoparticle formation rate;
- elimination of time-consuming purification steps such as centrifugation or filtration.

Electrochemical characterisation using CV, DPV and EIS confirmed the enhanced electrocatalytic properties of the AuNPs-modified electrodes, attributed to improved ET efficiency and increased surface conductivity.

Functionalisation with 6-ferrocenyl-hexanethiol via the same ESI microdroplet deposition technique also resulted in superior redox behaviour compared to conventional self-assembled monolayer (SAM) methods.

The ESI technique allowed for a specific interaction between the 6-ferrocenyl-hexanethiol and the AuNPs, which promoted an effective redox activity while minimising non-specific adsorption.

Moreover, deposition and functionalization of the AuNPs through charged microdroplets deposition may occur in two consecutive steps of a few minutes without any electrode surface manipulation, saving processing time and improving the uniformity and repeatability of the modified layer.

Finally, the platform at 10 min of microdroplets deposition showing the best electrochemical performance was used to develop an electrochemical sensor for H_2O_2 detection.

The device exhibited a linear range of 5–70 μM and a LOD of 0.9 μM .

These findings highlight the versatility and efficacy of ESI microdroplets deposition in fabricating and functionalizing nanostructured electrodes, paving the way for advanced electrochemical sensors and biosensors.

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8. Conclusions

This thesis explores the exceptional potential of ionic reactions in confined volumes, utilizing electrospray ionization mass spectrometry (ESI) both as an analytical tool and as a method for chemical synthesis.

The results demonstrate that molecular reactivity in microdroplets and thin films generated by microdroplet deposition significantly surpasses that of traditional bulk methods.

These methodologies stand out for their ability to accelerate reactions, enhance selectivity, and reduce resource consumption, positioning themselves as sustainable and innovative solutions for synthetic chemistry.

The introduction of ESI, awarded the Nobel Prize for its impact on mass spectrometry, has revolutionized not only chemical analysis but also synthesis under ambient conditions.

As described in Chapter 1, the charged microdroplets created by ESI provide a unique reactive environment characterized by a high surface-to-volume ratio, partial desolvation of reagents, and localized increases in concentration and pH due to solvent evaporation.

These factors, combined with the charge accumulation at the gas-liquid interface, create kinetic conditions that dramatically accelerate reactions compared to bulk processes.

A central theme emerging from the studies reported in this thesis is the significant acceleration of reactions in confined volumes compared to bulk. Processes that would require hours or high temperatures in solution can be completed in few seconds within microdroplets or thin film generated by their deposition onto a target surface.

The results of my PhD research activity demonstrated the versatility of studies of reactions in microdroplets and thin film across a wide range of applications, from stereoselective reactions to electrochemistry.

Despite the promising results, these studies also highlight future challenges, particularly in optimizing operational conditions and scaling these technologies for industrial use.

Reactions in confined volumes redefine the landscape of synthetic chemistry proposing advanced and green methodologies to tackle the challenges of the 21st century.

The acceleration of reactions, combined with sustainability and innovation, represents not only a scientific milestone but also an opportunity for a more sustainable and technologically advanced future.

Candidate List of Publications

- 1) Salvitti, C.; de Petris, G.; Troiani, A.; **Managò, M.**; Villani, C.; Ciogli, A.; Sorato, A.; Ricci, A.; Pepi, F. Accelerated d-Fructose Acid-Catalyzed Reactions in Thin Films Formed by Charged Microdroplets Deposition. *J. Am. Soc. Mass. Spectrom.* **2022** Mar 2;33(3):565-572. DOI: 10.1021/jasms.1c00363.
- 2) Salvitti, C.; Pepi, F.; **Managò, M.**; Bortolami, M.; Michenzi, C.; Chiarotto, I.; Troiani, A.; de Petris, G. Free N-heterocyclic carbenes from Brønsted acidic ionic liquids: Direct detection by electrospray ionization mass spectrometry. *Rapid. Commun. Mass. Spectrom.* **2022** Sep 15;36(17):e9338. DOI: 10.1002/rcm.9338.
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