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**SMART POLYMER THIN FILMS: DESIGN,
DEVELOPMENT AND APPLICATIONS IN THE
FOOD INDUSTRY AND ENVIRONMENTAL
REMEDATION**

***THIN FILMS* DE POLÍMEROS INTELIGENTES: DISEÑO,
DESARROLLO Y APLICACIONES EN LA INDUSTRIA
ALIMENTARIA Y LA REMEDIACIÓN AMBIENTAL**

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RESUMEN

Esta memoria describe el diseño, desarrollo, caracterización y aplicación de films poliméricos como polímeros inteligentes. Tras una introducción que contextualiza la investigación y expone los problemas detectados y los objetivos planteados, se presentan dos grandes capítulos organizados según el área de aplicación de los polímeros inteligentes: la industria alimentaria y la remediación ambiental. En el ámbito alimentario se describen dos polímeros inteligentes destinados a la industria láctea: un polímero reactivo con enzimas ancladas para la obtención de leche sin lactosa y un polímero sensor para la cuantificación rápida y sencilla de proteínas en suero lácteo y en sus concentrados. Además, dentro de este mismo ámbito alimentario, se presentan dos polímeros sensores aplicados al análisis de betalaínas y capacidad antioxidante en remolachas. Por otro lado, en el ámbito de la remediación ambiental se describe un polímero inteligente reactivo con la enzima lacasa inmovilizada, desarrollado para la eliminación de bisfenol A (BPA) y de indigo carmín. Finalmente, se presenta un material capaz de detectar y eliminar nitratos en aguas de consumo.

ABSTRACT

This thesis describes the design, development, characterization, and application of polymeric films as smart polymers, specifically in the form of thin films. Following an introduction that contextualizes the research and outlines the identified challenges and objectives, two main chapters are presented, organized according to the application area of these smart polymers: the food industry and

environmental remediation. In the food sector, two smart polymers intended for the dairy industry are described: a reactive polymer with immobilized enzymes for the production of lactose-free milk, and a sensing polymer for the rapid and simple quantification of proteins in whey and whey concentrates. Additionally, within this same field, two sensing polymers applied to the analysis of betalains and antioxidant activity in beetroot are presented. On the other hand, in the field of environmental remediation, a smart reactive polymer with immobilized laccase is described, developed for the removal of Bisphenol A (BPA) and indigo carmine. Finally, a material capable of detecting and removing nitrates from tap water is presented.

CHAPTER 1

General introduction

Polymers are macromolecules present in virtually every area of life due to their great versatility, capable of adapting to the needs and challenges that society has faced over decades. Among the wide range of polymer applications, a relatively new and highly specific niche is that of smart polymers. These polymers are characterized by their ability to respond to external stimuli, either by carrying out a specific action, referred to as reactive polymers, or by producing a detectable and measurable signal, known as sensory polymers. Within this category of smart polymers, one of the possible physical formats is thin films, which have proven to be a robust and versatile option, capable of being tailored to application requirements and chemically modified through solid-phase reactions. In this context, this doctoral thesis focuses on the development of these smart polymers in their thin-film format for applications in the food industry and environmental remediation.

1.1. Fundamentals of polymers

1.1.1. Structure and basic classification of polymers

The term polymer comes from the Greek words *poly*, meaning “many,” and *meros*, meaning “part.” Each of these parts is called a monomer, from *monos* (“single”) and *meros* (“part”). In other words, a polymer can be defined as a large molecule made up of many repeating monomer units, which are the basic building blocks of this polymeric chain. Polymers can be classified from several points of view, depending on their origin, chemical structure, functionality, or morphology.¹

According to their source of origin, polymers can be divided into natural and synthetic. Natural polymers occur in nature and play essential roles in biological systems. Molecules such as DNA, cellulose, or proteins (including

¹ T. A. Saleh, *Polymer Hybrid Materials and Nanocomposites*, William Andrew Publishing, Elsevier, 2021.

enzymes) belong to this group.² In contrast, synthetic polymers are produced in laboratories or industries through chemical reactions involving synthetic monomers. These materials have been used for a long time in daily life, with well-known examples shown in Figure 1, including polytetrafluoroethylene (PTFE), used in Teflon® nonstick cookware, its expanded form (ePTFE) employed in water-proof Gore-Tex® fabrics, poly(p-phenylene terephthalamide (PPTA), also known as Kevlar®, used in bullet-resistant materials, polymethyl methacrylate (PMMA), used in car headlights and lenses, and polyvinylpyrrolidone (PVP), used in povidone-iodine (Betadine®) and hair sprays.

Based on their polymeric structure, polymers can be categorized as linear, branched, or cross-linked. Linear polymers consist of long chains of monomers arranged in a straight configuration, without covalent bonds between chains, instead, they are held together by weak van der Waals or hydrogen interactions. Branched polymers, on the other hand, possess a main chain with side branches that emerge from different points along the backbone. Cross-linked polymers are formed when chains are covalently connected through cross-linking agents, resulting three-dimensional networks.³

According to their chemical composition, polymers can be classified as homopolymers or copolymers. Homopolymers are composed of only one type of monomer, whereas copolymers contain two or more different monomers. In the latter case, if the monomers are arranged in no specific order along the chain, the resulting material is known as a random copolymer. Other types also exist, such as alternating copolymers, where the monomers alternate in a regular sequence along the chain, and block copolymers, in which long sequences of each monomer are grouped together to form distinct blocks.⁴

² A. Aravamudhan, D. M. Ramos, A. A. Nada, S. G. Kumbar, *Natural and Synthetic Biomedical Polymers*, 2014, 67–89.

³ J. Chen, E. S. Garcia, S. C. Zimmerman, *Accounts of Chemical Research*, 2020, 53, 6, 1244–1256.

⁴ S. L. Perry, C. E. Sing, *ACS Macro Letters*, 2020, 9, 2, 216–225.

Having discussed this classification, it is important to note that the polymers used in this thesis exhibit three key characteristics: they are synthetic, crosslinked, and random copolymers.

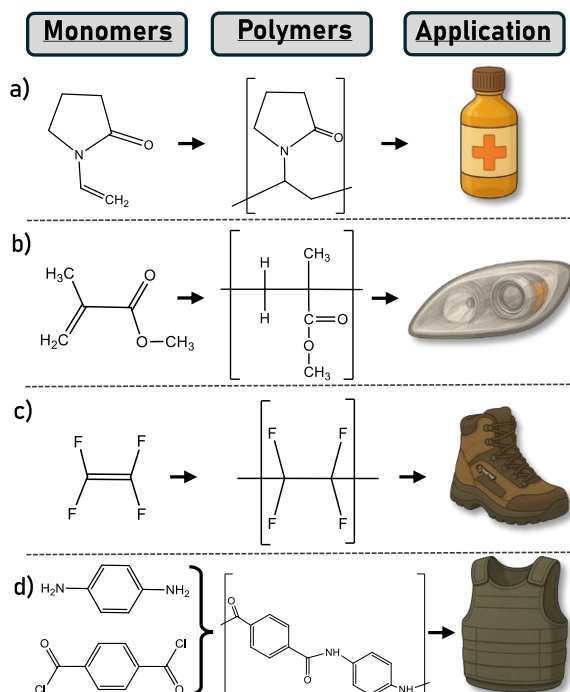


Figure 1. Examples of synthetic monomers, their derived polymers, and real-world applications: polyvinylpyrrolidone (PVP) used in povidone–iodine solutions (a); polymethyl methacrylate (PMMA) used in car headlights (b); expanded polytetrafluoroethylene (ePTFE) used in waterproof footwear (c); and Kevlar® used in bullet-resistant clothing (d).

1.1.2. Applications of polymers

The field of polymeric materials is undeniably one of the most dynamic and prolific areas of modern research. Its intense activity is reflected in a vast body of literature, with thousands of scientific articles published on the topic over several decades. This wide range of materials can be classified by their real-world application into three big groups:

- **Commodity polymers:** These are the most common and widely used polymers in daily life. They are employed in packaging, food containers, household products, and construction materials, among many others. Examples: polyethylene (PE), polypropylene (PP), polyvinyl chloride (PVC), and polystyrene (PS).⁵
- **Engineering polymers:** These polymers maintain their mechanical and chemical stability under demanding conditions such as high temperature, pressure, or corrosion. They are typically used in aerospace, automotive, and protective textile industries. Examples: polyamide 6.6 (Nylon 6.6), poly(m-phenylene terephthalamide) (Nomex®), poly(p-phenylene terephthalamide) (Kevlar®), polycarbonate (PC), and polytetrafluoroethylene (PTFE or Teflon®).⁶
- **High value-added polymers:** These are specialized materials designed for specific or advanced applications and are generally produced in smaller quantities. This group includes conductive, antimicrobial, antioxidant, and biopolymeric materials, as well as smart polymers.⁷

It is within this latter class of **smart polymers** that the work presented in this thesis is focused. These materials, capable of responding to external stimuli, represent a growing area of research with promising applications in biomedicine,⁸ food science,⁹ and environmental remediation.¹⁰

⁵ A. L. Andradý, M. A. Neal, *Philosophical Transactions of the Royal Society B: Biological Sciences*, 2009, 364, 1977-1984.

⁶ K. M. Praveen, C. V. Pious, S. Thomas, Y. Grohens, Relevance of Plasma Processing on Polymeric Materials and Interfaces, in: *Non-Thermal Plasma Technology for Polymeric Materials: Applications in Composites, Nanostructured Materials, and Biomedical Fields*, Elsevier, 2019, pp. 1–21.

⁷ D. Thakkar, R. Sehgal, A. K. Narula, D. Deswal, *Chemical Communications*, 2024, 61, 192–206,

⁸ M. Hrubý, S. K. Filippov, P. Štěpánek, *European Polymer Journal*, 2015, 65, 82–97.

⁹ J. Gómez-Estaca, C. López-de-Dicastillo, P. Hernández-Muñoz, R. Catalá, R. Gavara et al., *Trends in Food Science & Technology*, 2014, 35, 42–51,

¹⁰ H. N. Gray, D. E. Bergbreiter, *Environmental Health Perspectives*, 1997, 105, 55-63.

1.2. Smart polymers

In recent decades, polymer science has advanced beyond the creation of simple structural materials to the design of macromolecules with increasing complexity in their architecture and function. A prime example of this evolution is the emergence of smart polymers. These are materials engineered to exhibit a detectable, measurable response to specific changes in their environment. The ability to classify these materials is crucial and is typically based on three criteria: the nature of the stimulus that triggers the change, the mechanism that drives the transformation, and the resulting output response (Figure 2).¹¹

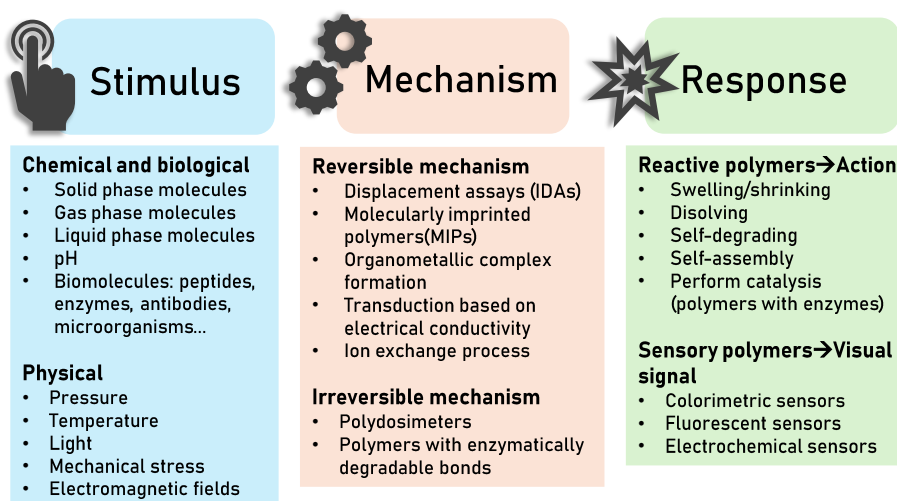


Figure 2. Graphical abstract of the classification of smart polymers based on the nature of the stimuli they respond to, their underlying mechanisms, and their resulting responses.

Regarding the **type of stimuli**, two main groups can be distinguished:

- **Chemical and biological stimuli:** these polymers respond to single molecules in the solid, liquid, or gaseous state, as well as macromolecules such as peptides, enzymes, antibodies,

¹¹ J. M. García, F. C. García, S. Vallejos, M. Trigo, J. A. Reglero, *Smart Polymers: Principles and Applications*, De Gruyter, 2022.

microorganisms, or even whole cells. These stimuli, often referred to as targets, are typically associated with a specific problem or condition. Their applications range from simple monitoring tasks, such as measuring pH levels in swimming pools, to more complex uses in solution, such as the detection of virus proteases in biological media or eliminating pollutants in drinking water.¹²⁻¹⁴

- **Physical stimuli:** these polymers are less common and respond to external physical factors such as pressure, temperature, mechanical stress, electromagnetic fields or light. Examples include photosensitive polymers, which undergo structural or chemical changes upon light irradiation and are widely used in drug delivery; thermochromic polymers, which exhibit reversible color changes in response to temperature variations and are used in devices to control room temperature;¹⁵ and piezoelectric polymers, capable of converting mechanical pressure into electrical signals and commonly used for human motion monitoring.¹⁶

Regarding the **mechanism of interaction** between the stimuli and the receptors in the polymer, two main groups can be distinguished: reversible and irreversible.

Among **reversible interactions**, four main mechanisms can be distinguished, some of which are schematically illustrated in Figure 3:

- **Displacement assays:** the polymer receptor initially binds a molecule that is released into the surrounding medium upon exposure to the stimulus (Figure 3a). This released molecule can serve as an indicator,

¹² S. Dai, P. Ravi, K. C. Tam, *Soft Matter*, 2008, 4, 435–449

¹³ A. Arnaiz, J. C. Guirado-Moreno, M. Guembe-García, R. Barros, J. A. Tamayo-Ramos, N. Fernández-Pampín, J.M García, S. Vallejos, *Sensors and Actuators B: Chemical*, 2023, 379, 133165

¹⁴ G. Utzeri, J. C. Guirado-Moreno, T.F.G.G. Cova, A. A. A. C. Pais, L. A. E. Batista De Carvalho, S. Ibeas, J. M. García, A. J. M. Valente, S. Vallejos, *npj Clean Water*, 2024, 7, 39.

¹⁵ A. Seeboth, D. Löttsch, *Encyclopedia of Polymer Science and Technology*, John Wiley & Sons, 2003.

¹⁶ T. Wang, M. Farajollahi, Y. S. Choi, I-T. Lin, J. E. Marshall, N. M. Thompson, S. Kar-Narayan, J. D. W. Madden, S. K. Smoukov, *Interface Focus*, 2016, 6, 20160026.

producing a visible signal in indicator displacement assays (IDAs), or as a therapeutic agent in drug delivery systems (DDS).¹⁷

- **Molecularly imprinted polymers (MIPs):** these polymers are synthesized in the presence of the target molecule, which acts as a template. After polymerization, the target is removed, leaving behind cavities complementary in shape and size, conferring high selectivity for rebinding the target in subsequent applications.^{18,19}
- **Organometallic complex formation:** in this mechanism, metal ions interact with polymer receptors, forming stable complexes.²⁰
- **Transduction based on electrical conductivity:** when the target binds to the polymer receptors, it induces a change in the polymer's conductivity. Examples of such polymers include polyaniline and polyacetylene.²¹
- **Ion exchange processes:** In this mechanism, ions are initially coupled to the receptors within the polymer. However, when the target ion is present, it displaces the pre-bound ions, leading to a change in the polymer's behaviour (Figure 3b).²²

Among **irreversible interactions**, two main groups, illustrated in Figure 3, can be distinguished:

- **Polydosimeters:** these polymers undergo an irreversible chemical reaction between the receptor groups in the polymer and the target. Once the interaction occurs, the polymer undergoes a structural change that is macroscopically measurable (Figure 3c). An example of this is covalent azo coupling reactions, which can take place between diazonium groups

¹⁷ A. C. Sedgwick, J. T. Brewster II, T. Wu, X. Feng, S. D. Bull, X. Qian, J. L. Sessler, T. D. James, E. V. Anslyn, X. Sun, *Chemical Society Reviews*, 2021, 50, 9-38.

¹⁸ N. Leibl, K. Haupt, C. Gonzato, L. Duma, *Chemosensors*, 2021, 9, 123.

¹⁹ J. A. García-Calzón, M. E. Díaz-García, *Sensors and Actuators B: Chemical*, 2007, 123, 1180–1194.

²⁰ J. W. Steed, *Chemical Society Reviews* 38, 2009, 506–19.

²¹ X. Jiang, H. Wang, R. Yuan, Y. Chai, *Analytical Chemistry*, 2018, 90, 8462–8469.

²² J. L. Vallejo-García, R. Hernández-Ruiz, A. Torija-López, M. Trigo-López, S. Ibeas, L. Gómez-Cuadrado, S. Martel, R. Barros, S. Vallejos, *Environmental Technology & Innovation*, 2025, 40, 104595.

in polymer receptors and a nucleophilic functional group in the target molecule.²³

- **Polymers containing enzymatically degradable bonds:** these polymers typically operate based on Förster resonance energy transfer (FRET). The system consists of a donor molecule (fluorophore or chromophore) and an acceptor (quencher) that suppresses emission under normal conditions. When the target interacts with the polymer, the FRET pair is cleaved enzymatically, resulting in a detectable visual or fluorescent change (Figure 3d).²⁴

Finally, regarding the **type of response**, two main groups can be distinguished:

- **Reactive polymers:** these smart polymers perform a specific action when the stimulus is present. For example, they can induce a drastic change in their structure, such as shrinking, swelling, dissolving, self-degrading and self-assembling, or perform catalysis when the target is present, for instance polymers with immobilized enzymes. Specific examples include drug delivery systems based on pH-sensitive polymers, which release drugs under acidic conditions in the affected area,²⁵ or polymers with immobilized enzymes capable of degrading pollutants.²⁶
- **Sensory polymers:** these smart polymers respond to a stimulus with a detectable and measurable signal, such as a color change (colorimetric sensors),²⁷ fluorescence emission (fluorescent sensors),²⁸ or a variation

²³ S. E. Bustamante Fonseca, B. L. Rivas, J. M. García Pérez, S. Vallejos Calzada, F. García, *Journal of Applied Polymer Science*, 2018, 135, 46185.

²⁴ R. Duncan, H. C. Cable, J. B. Lloyd, P. Rejmanová, J. Kopečekuth, *Die Makromolekulare Chemie*, 1983, 184.

²⁵ C. M. Wells, M. Harris, L. Choi, V. P. Murali, F. D. Guerra, J. A. Jennings, *Journal of Functional Biomaterials*, 2019, 10(3), 34.

²⁶ D. Gurgel, R. Oliveira Henriques, M. Kelbert, V. Conradi, B. F. Oechsler, A. F. Junior, D. de Oliveira, *Journal of Environmental Chemical Engineering*, 2025, 13, 116677.

²⁷ T.-T. Wang, C. K. Lio, H. Huang, R.-Y. Wang, H. Zhou, P. Luo, L.-S. Qing, *Talanta*, 2020, 206, 120211.

²⁸ S. Vallejos, E. Hernando, M. Trigo, F. C. García, M. García-Valverde, D. Iturbe, M. J. Cabero, R. Quesada, J. M. García, *Journal of Materials Chemistry B*, 2018, 22, 3735–3741.

in electrochemical properties (electrochemical sensors).²⁹ This alert is a direct consequence of the interaction between the target and the polymer receptor and can be recorded and quantified using UV–Vis spectrophotometers, fluorimeters, potentiostats or even smartphones. Such specific sensors are widely applied in diverse fields, including biomedicine, environmental monitoring, and the food industry.

Smart polymers can be found in various physical formats, such as fibers, membranes, gels, beads or spherical particles, and films. Among this diversity, the present thesis will focus on thin films, which themselves can exist in different configurations and offer distinct functional advantages. These thin polymer films will be introduced and discussed in detail in the following section.

²⁹ M. A. Rahman, P. Kumar, D.-S. Park, Y.B. Shimd, *Sensors*, 2008, 8, 118-141.

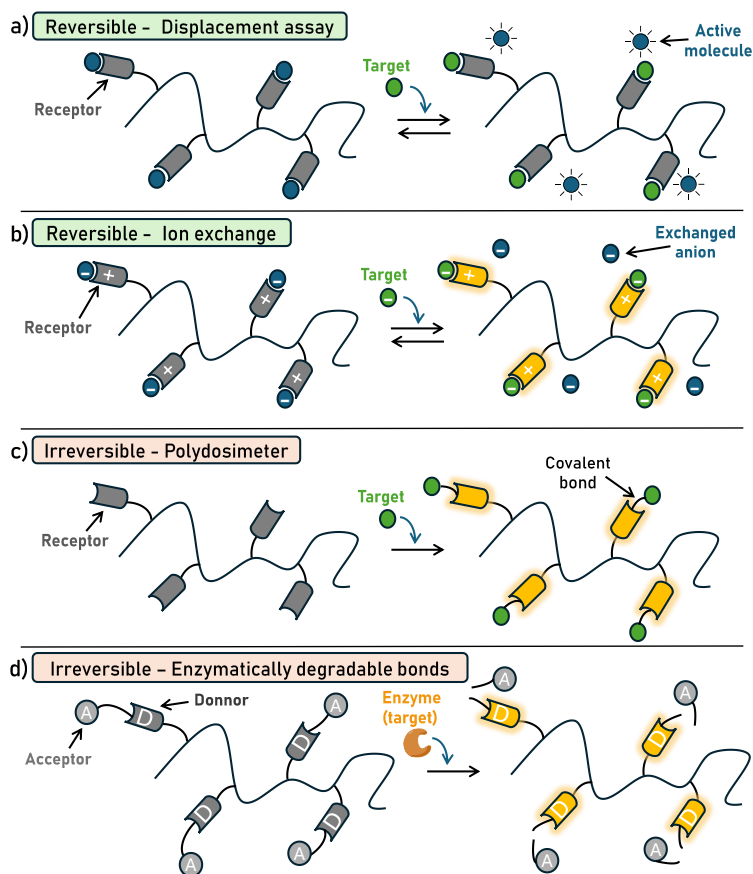


Figure 3. Schematized examples of reversible and irreversible mechanisms in smart polymers.

1.3. Thin films as smart polymers

Thin films are layers of materials whose thickness ranges from a few nanometers to tens of micrometers. These materials are at the forefront of smart polymers due to their excellent properties, which are often superior to those of their conventional bulk counterparts, allowing precise control of functional properties and exhibiting unique physical and chemical functionalities.

Their study dates back to the 16th century, when scientists observed reflective effects in thin Ag metallic thin layers on glass, but it was in the 20th century that advances in deposition techniques such as vacuum evaporation, sputtering, and chemical vapor deposition enabled precise control over film composition, thickness, and structure.³⁰ Thin films quickly found applications in electronics, including transistors and integrated circuits, optics for anti-reflective coatings and mirrors, protective coatings for wear and corrosion resistance, and biomedical devices such as temporary implants and controlled drug delivery systems.³¹⁻³⁴ More recently, thin films have also been used as smart polymers engineered to respond to physical, chemical or biological stimuli in a controlled manner. Thin films can be organic or inorganic. In this thesis, organic synthetic films were used, providing tuneable chemical composition, surface functionality, and structural properties suitable to work as smart polymers.

Traditionally, thin polymeric films were obtained by solution casting, a process in which a pre-synthesized polymer is dissolved, deposited onto a surface, the solvent is evaporated, and the resulting film is then peeled off.³⁵ However, in this thesis, the focus will be on freestanding thin films that do not require a substrate to maintain their structural integrity. In this case, the polymerization occurs directly within a mold, leading to the in-situ formation of the film without intermediate steps such as dissolution, processing, or shaping. This in-mold approach enables precise control over film thickness, homogeneity, and composition, and produces materials that are mechanically stable and self-supporting. Moreover, these in-mold polymerized thin films can subsequently undergo solid-phase post-reactions to introduce functional groups or immobilize

³⁰ S. Sakthinathan, G. A. Meenakshi, S. Vinothini, C.-L. Yu, C.-L. Chen, T.-W. Chiu, N. Vittayakorn, *Processes*, 2025, 13(2), 587.

³¹ P. Wang, X. Fu, D. Gibson, L. Fleming, S. Ahmadzadeh, C. Li, M. Muhiyudin, S. Song, D. Hutson, D. Moodie, C. MacGregor, M. S. P Wang, *Coatings*, 2018, 8(12), 472

³² J. Vetter, *Surface and Coatings Technology*, 2014, 257, 213–240.

³³ V. L. S. dos Santos, R. C. Araújo, E. S. Lisboa, A. M. Lopes, R. L. de Albuquerque-Júnior, J. C. Cardoso, C. Blanco-Llamero, T. A. Deshpande, H. O. W. Anderson, R. Priefer, E. B. Souto, P. Severino, *Journal of Drug Delivery Science and Technology*, 2024, 9, 105243.

³⁴ L. W. Hrubesh, J. F. Poco, *Journal of Non-Crystalline Solids*, 1995, 188, 46–53.

³⁵ K. Shuto, Y. Orsm, T. Kajiyama, C. C. Han, *Polymer Journal*, 1993, 25, 291–300.

molecules or macromolecules, further enhancing their performance and versatility.

In this work, the term thin film therefore specifically refers to this freestanding, in-mold polymerized material, which provides an ideal platform for both reactive and sensory smart polymer application.

1.3.1. Thin film formats

Thin films can adopt various structural and physical formats, which strongly influence the interaction between the polymer and target molecules. These formats can be classified as follows:

- **Smooth surface dense films:** these films are prepared in-mold, by injecting a solution containing the monomers between two silanized glass plates, separated by a mold that defines the desired thickness (typically between 10 and 200 μm). This configuration minimizes thickness heterogeneity and simplifies the fabrication process, yielding uniform, self-supporting dense films.³⁶
- **Rough surface dense films:** these materials are prepared following the same procedure as smooth dense films but using textured glass molds. The glasses employed are similar to those used for shower screens, transferring their microscale roughness to the polymer surface during polymerization. The resulting surface increases the effective surface area of the smart polymer, thereby enhancing its interaction with target molecules.³⁷
- **Foam-based films:** in this case, the polymer films are produced through a foaming process using supercritical CO_2 . Pores are generated both on the surface and within the bulk of the film, leading to a porous architecture

³⁶ S. Vallejos, A. Muñoz, S. Ibeas, F. Serna, F. C. García, J. M. García, *Journal of Materials Chemistry A*, 2013, 1, 15435–15441.

³⁷ A.Arnaiz, B. Melero, M. Trigo, A. Mendiá, D. De La Fuente, M.E. Iñigo, L. Gómez, S. Ibeas, S. Vallejos, *Sensors and Actuators B: Chemical*, 2026, 451, 139378

with a significantly larger specific surface area, which greatly improves contact between the polymer and its target analytes.³⁸

- **Thin films from electrospun nanofibers:** these films are obtained by electrospinning, a process in which a high voltage is applied to a polymer solution extruded through a fine needle. The resulting nanofibers are collected onto a grounded collector, forming a three-dimensional nonwoven mat. This fibrous morphology provides a large accessible surface area and promotes efficient interaction between the smart material and the target molecules.³⁹
- **Porous thin films:** these materials are typically hydrogels, consisting of a three-dimensional network of crosslinked polymers capable of absorbing large amounts of water while maintaining structural integrity. This hydrated structure enhances mass transport and molecular recognition in aqueous media. A variation of these materials is the aerogel, obtained by removing the solvent from the gel pores without collapsing the 3D structure. Aerogels exhibit extremely high porosity (>90%) and low density, maximizing the surface area available for target interaction.⁴⁰
- **Layer-by-layer (LbL) assembled thin films:** These films are formed through the sequential adsorption of oppositely charged polymers or biomolecules, where each deposition step adds a nanometric layer via electrostatic or hydrogen-bonding interactions. Although they are commonly built on solid substrates such as glass or electrodes, LbL films can also be freestanding. This versatile technique allows precise control over film thickness and composition, enabling their use as smart polymer systems.⁴¹

³⁸ J. A. Reglero Ruiz, S. Vallejos, A. M. Sanjuán, F. C. García, M. Múgica, M. Á. Rodríguez-Pérez, J. M. García, *Journal of Applied Polymer Science*, 2018, 135, 45872.

³⁹ A. Arnaiz, M. Guembe-García, M. Martínez, M. Trigo-López, E. González, I. Otsuka, S. Vallejos, *Sensors and Actuators B: Chemical*, 2025, 425, 136976.

⁴⁰ F. Pinelli, L. Magagnin, F. Rossi, *Materials Today Chemistry*, 2020, 17, 100317.

⁴¹ O. Cassidy, K. Synnatschke, J. M. Munuera, C. Gabbett, T. Carey, L. Doolan, E. Caffrey, J. N. Coleman, *Npj 2D Materials and Applications*, 2025, 9, 2.

- **Thin films produced by coating:** Functionalized polymers can be applied as coatings onto different substrates. This approach is particularly useful when the properties of dense films are not suitable for the intended application, as it allows the use of a pre-synthesized substrate with excellent properties onto which the smart polymer can be deposited, generally in its linear form.⁴²

Having mentioned these formats, it is important to note that this thesis will focus exclusively on smooth surface dense thin films. Although this configuration may not provide the highest surface area or the most extensive contact with target molecules, it offers clear advantages in terms of mechanical robustness, homogeneity, and ease of handling, as well as a simplified and reproducible fabrication process. Moreover, this thin film format has been extensively studied and applied within our research group for nearly three decades, serving as a reliable platform for both reactive and sensory smart polymer systems.

1.3.2. Dense thin film design and preparation strategies: polymerization methods

Smart polymer thin films are obtained through conventional polymerization methods, which can be classified into two main groups:

- **Addition polymers** are produced by chain-growth reactions, since the monomers do not contain reactive functional groups capable of reacting with each other. For this reason, an initiator is required and no small-molecule by-products are generated. This type of polymerization involves vinyl monomers and proceeds through three stages: initiation, propagation, and termination. Depending on the type of initiator used, the process can follow a radical mechanism (thermal, photochemical, or redox) or an ionic mechanism (cationic or anionic).⁴³ When two or more

⁴² R. Aguado, A. R. M. G. Santos, S. Vallejos, A. J. M. Valente, *Molecules*, 2022, 27(9), 2900.

⁴³ Laurence W. McKeen, *Introduction to Plastics and Polymers*, in: *The Effect of Long Term Thermal Exposure on Plastics and Elastomers*, William Andrew, 2014, pp. 1–16.

different monomers are used, random copolymers are obtained. This mechanism is illustrated in Figure 4a.

- **Condensation polymers** are formed by the reaction of monomers bearing complementary functional groups, which can react without the need for an initiator. Typical examples include alcohols reacting with acids to form polyesters, or acids reacting with amines to generate polyamides. During these step-growth reactions, a small molecule, often water, is released.⁴⁴ This mechanism is illustrated in Figure 4b.

In this thesis, all polymers employed are random copolymers, obtained from vinyl monomers through addition polymerization.

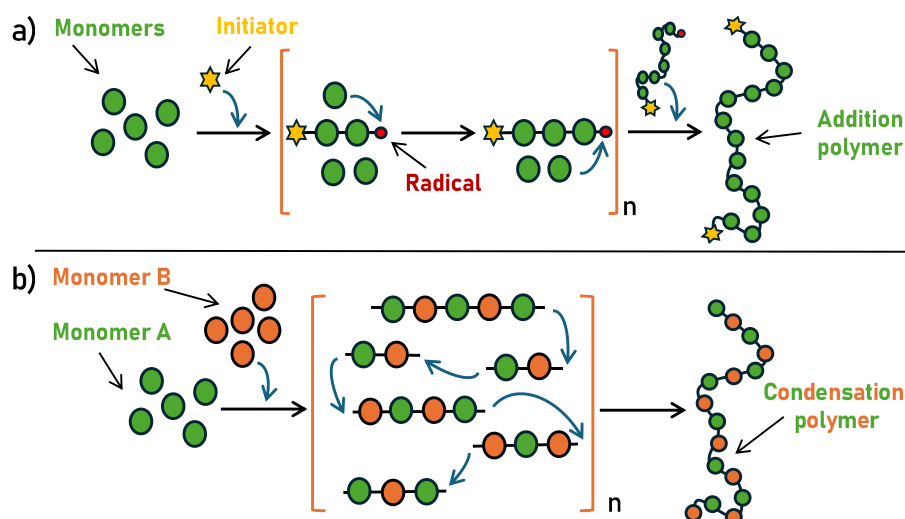


Figure 4. Graphical representation of polymerization mechanisms: addition polymerization (a) and condensation polymerization (b).

1.3.3. Dense thin film design and preparation strategies: chemical composition

From a chemical point of view, the general structure of the dense thin films smart polymers, prepared in this thesis, is typically composed of three main

⁴⁴ P. J. Flory, *Chemical Reviews*, 1946, 39, 1, 137–197.

components: the structural matrix, the functional or receptor groups, and the cross-linking agent (Figure 5).⁴⁵

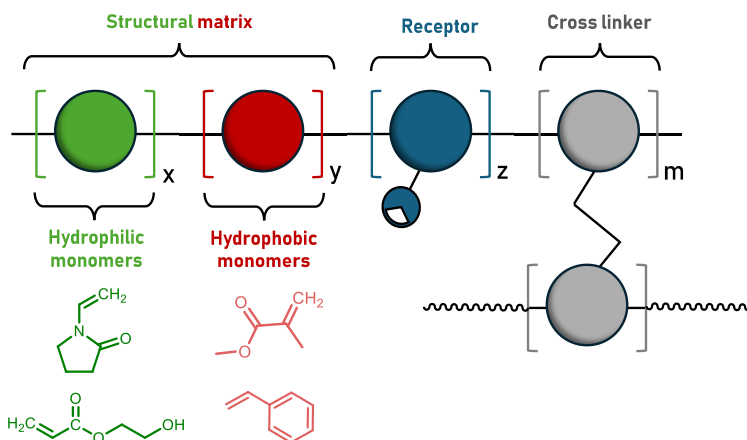


Figure 5. Simplified representation of the chemical structure of thin films.

The **structural matrix** consists of one or more monomers selected according to the desired material properties. It usually represents more than 90% of the total monomer composition, and therefore plays a decisive role in determining the overall characteristics of the polymer. Among the most relevant properties influenced by the matrix monomers are:

- **Hydrophilicity/Hydrophobicity:** This refers to the affinity of the polymer film for water. Hydrophilic films can absorb water and swell, which is particularly important for smart polymers, as they often operate in aqueous media where swelling facilitates the interaction between target molecules and receptor sites. Monomers like *N*-vinylpyrrolidone,^{46,47} 2-hydroxyethyl acrylate,⁴⁸ and acrylamide,⁴⁹ impart hydrophilicity whereas

⁴⁵ S. Vallejos, J. A. Reglero, F. C. García, J. M. García, *Journal of Materials Chemistry A*, 2017, 5, 13710–13716.

⁴⁶ P. Franco, I. De Marco, *Polymers*, 2020, 12(5), 1114.

⁴⁷ F. Haaf, A. Sanner, F. Straub, *Polymer Journal*, 1985, 17, 143–152.

⁴⁸ Y. Cui, X. Jiang, C. Feng, G. Gu, J. Xu, X. Huang, *Polymer Chemistry*, 2016, 7, 3156–3164.

⁴⁹ L. Hanyková, J. Štátná, I. Krakovský, *Gels*, 2024, 10(7), 414.

methyl methacrylate,⁵⁰ styrene,⁵¹ and butyl acrylate,⁵² impart hydrophobicity to the resulting film.

- **Mechanical properties:** These define the behaviour of the polymer when subjected to deformation or stress. For example, methyl methacrylate, with its short side chain, contributes to rigidity and strength,⁵³ while lauryl methacrylate, with a longer alkyl chain, provides flexibility. Likewise, *N*-vinylpyrrolidone contributes rigidity to the polymer network, whereas 2-hydroxyethyl acrylate enhances flexibility.⁵⁴

The **receptor** unit corresponds to the functional monomer capable of interacting with the target molecule and generating a response, either in the form of an action or a measurable signal. It is generally present in amounts below 10% of the total monomer composition.⁵⁵

Finally, the **cross-linking agent** acts as a molecular “bridge” that interconnects polymer chains, preventing dissolution of the film in solvents. It is typically included in small proportions (below 1%) and is added as an additional monomer during the copolymerization process. The cross-linker also plays a crucial role in determining the material's behavior in aqueous environments: a lower cross-linking degree allows greater chain mobility and consequently a higher swelling capacity in water, whereas a higher cross-linking percentage restricts chain movement, leading to reduced swelling.⁵⁶

The key to obtain high-performance smart polymers lies in balancing hydrophilicity with mechanical strength, ensuring stability in both the dry and swollen state. Generally, increasing the hydrophilic character of the material

⁵⁰ B. Ersoy, H. Çiftçi, A. Evcin, Y. Merdivenci, *Physicochemical Problems of Mineral Processing*, 2022, 58(5):152162

⁵¹ A. Faghihnejad, H. Zeng, *Soft Matter*, 2012, 8, 2746-2759.

⁵² M. Abdollahi, H. Khakpour, *RSC Advances*, 2015, 5, 102844-102855.

⁵³ S. S. Rahman, M. B. Mahmud, A. R. Monfared, P. C. Lee, C. B. Park, *Composites Science and Technology*, 2023, 236, 109994.

⁵⁴ D. Aki, M. Esmaeili Rad, E. C. Şenel, M. C. Onceyiz, M. C. Gokmenoglu, L. Duta, O. Gunduz, *Biomedical Materials*, 2025, 20, 045026

⁵⁵ J. M. García, F. C. García, F. Serna, J. L. de la Peña, *Polymer Reviews*, 2011, 51, 341-390

⁵⁶ C. W. H. Rajawasam, O. J. Dodo, M. A. S. N. Weerasinghe, I. O. Raji, S. V. Wanasinghe, D. Konkolewicz, N. De Alwis Watuthantrige, *Polymer Chemistry*, 2024, 15, 219-247.

enhances its interaction with the target molecules; however, if the hydrophilicity is too high, the mechanical properties of the polymer may be compromised. Conversely, increasing the proportion of hydrophobic monomers improves the mechanical robustness of the film but reduces its responsiveness in aqueous media. To ensure an optimal balance between these two properties, equimolar mixtures of *N*-vinylpyrrolidone (NVP) and methyl methacrylate (MMA) have been employed in the course of this thesis. These monomers have been selected because their corresponding polymers PVP and PMMA, are known to be biocompatible, chemically and thermally stable, exhibit low toxicity and minimal migration once polymerized, and are commercially available and cost-effective, which are crucial characteristics for their application in food-related industries, environmental remediation and biomedical fields.⁵⁷⁻⁵⁹

1.3.4. Dense thin film design and preparation strategies: receptor incorporation

Depending on how this receptor is incorporated into the polymeric matrix, two main strategies can be distinguished:

- **Synthesis of the receptor followed by copolymerization:** in this approach, the receptor monomer is first synthesized through conventional chemical reactions (Figure 6a). The receptor must contain well-defined chemical moieties designed to selectively interact with the target analyte, as well as a polymerizable functional group, such as a vinyl or methacrylate group, which enables its copolymerization with other structural monomers during film formation. This strategy ensures a

⁵⁷ M. S. Pérez-Garibay, G. Á. Lara-Rodríguez, E. Bucio, *Gels*, 2025, 11(2), 147.

⁵⁸ S. M. Pituru, M. Greabu, A. Totan, M. Imre, M. Pantea, T. Spinu, A. M. C. Tancu, N. O. Popoviciu, I.-I. Stanescu, E. Ionescu, *Materials*, 2020, 13(13), 2894.

⁵⁹ A. Abdullah, J. Cai, M. A. Hafeez, Q. Wang, S. Farooq, Q. Huang, W. Tian, J. Xiao, *Frontiers in Nutrition*, 2022, 9.

uniform and stable incorporation of the receptor within the polymer network, preventing the receptor migration out of the material.⁶⁰

- **Copolymerization followed by solid-phase chemical modification:** In this approach, the thin film is first synthesized through the polymerization of monomers, after which solid-phase reactions are carried out to modify the chemical structure of specific monomer units, introducing the desired functional groups that will act as receptors (Figure 6b). This method offers several advantages, as it avoids the inherent limitations and time-consuming steps of conventional organic synthesis, such as solution-phase reactions, column purification procedures, and the use of large solvent volumes. A remarkable example of this approach is the incorporation of 4-aminostyrene into the structural matrix, which can subsequently be transformed via solid-phase diazotization into aryl diazonium salts. These diazonium groups can undergo azo coupling reactions in the presence of specific targets such as nitrites, phenols, or proteins, resulting in a visible color change in the polymer.^{61,62}

Furthermore, it is worth mentioning that solid-phase reactions in films have proven highly effective, as the polymer matrix provides a favorable environment for stabilizing intermediates.

⁶⁰ J. C. Guirado-Moreno, L. González-Ceballos, I. Carreira-Barral, S. Ibeas, M. A. Fernández-Muiño, M. T. Sancho, J. M. García, S. Vallejos, *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 2023, 284, 121820.

⁶¹ L. González-Ceballos, M. del M. Cavia, M. A. Fernández-Muiño, S. M. Osés, M. T. Sancho, S. Ibeas, F. C. García, J. M. García, S. Vallejos, *Food Chemistry*, 2021, 342, 128300.

⁶² J. C. Guirado-Moreno, M. Guembe-García, J. M. García, R. Aguado, A. J. M. Valente, S. Vallejos, *ACS Applied Materials and Interfaces*, 2021, 13, 50, 60454-60461.

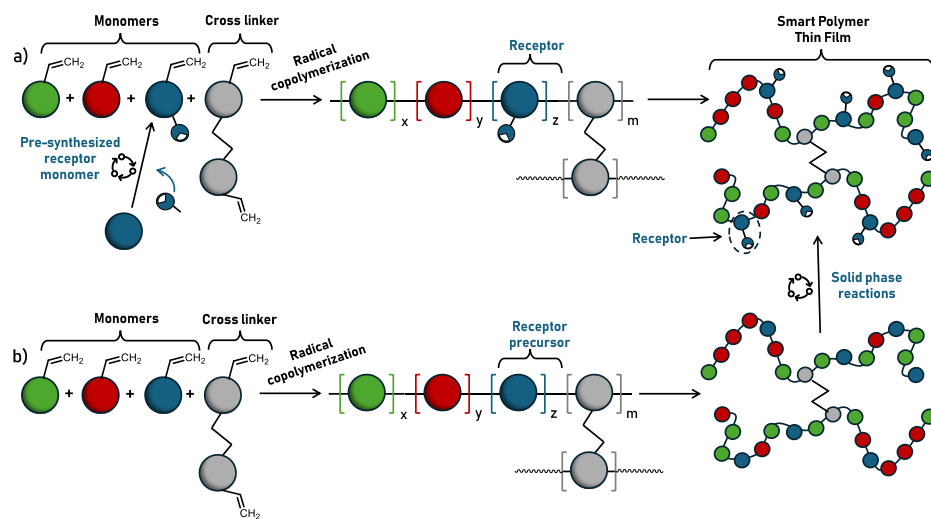


Figure 6. Simplified representation of receptor incorporation into thin films: synthesis of the receptor followed by copolymerization (a), copolymerization followed by solid-phase chemical modification (b).

1.4. Thin films as reactive polymers with immobilized enzymes

Enzymes are proteins that carry out biochemical reactions in living organisms. Structurally, they are polypeptides, since they are composed of chains of amino acid monomers whose sequence and spatial arrangement give rise to a unique three-dimensional structure essential for their catalytic function. Enzymes are extremely efficient biocatalysts: a single enzyme molecule can convert thousands of substrate molecules per second. In addition, they exhibit remarkable specificity, typically catalyzing only one type of reaction or acting on a closely related group of substrates, ensuring precise biochemical control within cells.⁶³ These features, combined with the wide catalog of enzymes available, make them suitable for numerous applications in different industrial sectors, such as

⁶³ S. Jemli, D. Ayadi-Zouari, H. Ben Hlima, S. Bejar, *Critical Reviews in Biotechnology*, 2016, 36, 246–258,

the food industry, pharmaceuticals and medicine, bioenergy production, the textile and paper sector, and environmental biotechnology and bioremediation.⁶⁴

Traditionally, enzymes have been used in their free and soluble form in batch-type processes, where at the end of each cycle they must be separated from the product through denaturation, precipitation, or ultrafiltration, causing the enzyme to lose its catalytic function. As a result, new enzymes must be employed in each process cycle, considerably increasing industrial costs.⁶⁵

Enzyme immobilization addresses this limitation by fixing biocatalysts to a support, allowing their reuse and facilitating the separation of the product from the enzymes. Furthermore, immobilization can confer greater operational stability to the enzyme, making it more resistant to changes in pH and temperature, an important advantage in industrial applications where these conditions can vary and strongly affect final yields. Additionally, immobilization enables operation in continuous-flow bioreactors, which offer multiple benefits over batch or stationary processes, such as easier automation and elimination of non-productive downtime, resulting in significant cost savings.⁶⁶

However, immobilization may also present drawbacks arising from changes in the enzyme's protein structure induced by binding to supports. The three-dimensional conformation of an enzyme is crucial for its catalytic activity; thus, if the native structure is altered during immobilization, enzymatic activity could decrease. Another indirect effect to consider is the microenvironmental impact, which may lead to heterogeneous solute distribution between the enzyme's immediate surroundings and the bulk phase due to partitioning or limited diffusion. These effects, both positive and negative, depend on the properties of the support matrix, the immobilization method, and the nature of the

⁶⁴ J. Chapman, A. E. Ismail, and C. Z. Dinu, *Catalysts*, 2018, 8(6), 238.

⁶⁵ D. S. Clark, *Trends in Biotechnology* 12, 199, 11, 439–443.

⁶⁶ P. K. Robinson, *Essays in Biochemistry*, 59, 1–41.

enzyme itself, meaning that the consequences of immobilization can vary widely in each specific case.^{67,68}

Even if an immobilization method does not provide biochemical advantages and reduces enzymatic activity, the ability to reuse the enzyme over multiple cycles and easily separate it from the product can still be economically favourable compared to using free catalysts.

1.4.1. An overview of enzyme immobilization technologies in polymers

Immobilization methods can be broadly classified into two groups: physical and chemical. Figure 7 illustrates a schematic of the main methods within each group, described as follows:

1) Physical methods:

- **Adsorption:** In this method, the enzyme binds to the support via non-covalent interactions such as van der Waals forces, electrostatic interactions, hydrophobic interactions, or hydrogen bonds. The main advantage is simplicity and low cost, often preserving the enzyme's catalytic activity while allowing the support to be reused. However, since the interactions are weak, the enzyme may easily detach from the support under changes in pH, temperature, ionic strength, or mechanical stress.⁶⁹ Polymeric supports are commonly employed for adsorption-based immobilization. Among natural polymers, chitosan has been used to immobilize lipase,⁷⁰ while cellulose has served as a support for laccase.⁷¹ For synthetic polymers, PMMA has proven effective for

⁶⁷ Z. Ashkan, R. Hemmati, A. Homaei, A. Dinari, M. Jamlidoost, A. Tashakor, *International Journal of Biological Macromolecules*, 2021, 168, 708–721

⁶⁸ I. Karume, *Sustainable Chemistry for the Environment*, 2023, 4, 100048.

⁶⁹ D. M. Liu, J. Chen, Y. P. Shi, *TrAC Trends in Analytical Chemistry*, 2018, 102, 332–42.

⁷⁰ D. Pirozzi, A. Latte, F. Sannino, *Gels*, 2023, 9(10), 776.

⁷¹ R. Wu, F. Liu, Q. Dong, Y. Huang, Y. Qiu, Y. Sun, E. Su, *Applied Biochemistry and Biotechnology*, 2021, 193(2), 446–462.

horseradish peroxidase (HRP) immobilization,⁷² and nylon 6 has been successfully applied for laccase through adsorption.⁷³

- **Entrapment:** This includes entrapment in gels or fibers and microencapsulation. Gel or fiber entrapment physically confines the enzyme within a solid matrix. In microencapsulation, the enzyme is enclosed within a particle, usually spherical, creating a semi-permeable barrier that allows substrate entry and product exit while retaining the enzyme.⁷⁴ The main advantage of these methods is that the enzyme does not form covalent bonds with the support, thus maintaining its native conformation and catalytic activity. Additionally, these methods are simple, cost-effective, and allow easy preparation of multi-enzyme systems. Natural polymers are widely used in this type of immobilization. Examples include alginate, which has been used to immobilize β -galactosidase,⁷⁵ pectinases,⁷⁶ or invertases,⁷⁷ and κ -carrageenan, which has been used with lipases⁷⁸ or amylases.⁷⁹ Gel-type synthetic polymers can also be used, such as polyacrylamide, which has been employed with pectinases⁸⁰ and tyrosinases,⁸¹ polyethylene glycol (PEG) for immobilizing glucose oxidase,⁸² or polyvinyl alcohol (PVA) for β -galactosidase.⁸³ In this entrapment immobilization method, although they are not polymers, metal–organic frameworks are worth mentioning. They

⁷² S. Vorvi, K. Tsougeni, A. Tserepi, S. Kakabakos, P. Petrou, E. Gogolides, *Molecules*, 2024, 29(19), 4736.

⁷³ K. Jankowska, A. Grzywaczyk, A. Piasecki, E. Kijeńska-Gawrońska, L. N. Nguyen, J. Zdarta, L. D. Nghiem, M. Pinelo, T. Jesionowski, *Environmental Technology & Innovation*, 2021, 21, 101332.

⁷⁴ I. Eş, J. D. G. Vieira, A. C. Amaral, *Applied Microbiology and Biotechnology*, 2015, 99(5), 2065–2082.

⁷⁵ G. N. Martins, O. Figueira, P. C. Castilho, Immobilization of β -Galactosidase in Calcium Alginate Beads, in: *Basic Protocols in Encapsulation of Food Ingredients*, Humana Press, 2021: pp. 167–181.

⁷⁶ R. L. Oliveira, J. L. Dias, O. S. Silva, T. S. Porto, *Food and Bioproducts Processing*, 2018, 109, 9–18.

⁷⁷ I. Malhotra, S. F. Basir, *Preparative Biochemistry & Biotechnology*, 2020, 50(5), 494–503.

⁷⁸ K. R. Jegannathan, C. E. Seng, P. Ravindra, *Journal of Environmental Research And Development*, 2009, 4(2).

⁷⁹ M. G. Sankalia, R. C. Mashru, J. M. Sankalia, V. B. Sutariya, *International Journal of Pharmaceutics*, 2006, 312, 1–14.

⁸⁰ H. U. Rehman, M. A. Nawaz, S. Pervez, M. Jamal, M. Attaullah, A. Aman, S. A. U. Qader, *Heliyon*, 2020, 6(8).

⁸¹ J. G. Schiller, C. C. Liu, *Biotechnology and Bioengineering*, 1976, 18, 1405–1412.

⁸² D. Choi, W. Lee, J. Park, W. Koh, *Bio-Medical Materials and Engineering*, 2008, 18, 6.

⁸³ D. Akdoğan, A. Peksel, *Biotechnology and Applied Biochemistry*, 2025, 72, 652–663.

are widely used and are formed by transition metals and organic ligands, such as terephthalic acid or imidazole, offering high porosity and a large surface area.⁸⁴

2) Chemical methods:

- **Covalent binding:** This involves forming covalent bonds between functional groups on the support material and reactive residues of the enzyme. The main advantage is high stability, preventing enzyme leaching and increasing enzyme robustness for reutilization. However, chemical reactions can inactivate active sites, reducing catalytic activity.⁸⁵ There are numerous examples of synthetic polymers used, for instance poly(glycidyl methacrylate) (PGMA), which contains epoxy rings capable of reacting with the amino groups of enzymatic proteins, such as lipase,⁸⁶ or poly(styrene-co-maleic anhydride), which has been employed with lipase or trypsin.⁸⁷ There are also examples of polymers that are subsequently functionalized to react with the $-NH_2$ groups of enzymes, such as PMMA functionalized with EDC/NHS for immobilizing laccases,⁸⁸ nylon-6 functionalized with glutaraldehyde (GA) for lipase immobilization,⁸⁹ or aminated polystyrene resins also activated with GA, in this case for pectinases.⁹⁰
- **Cross-linking:** Also known as enzyme reticulation, this method involves covalent bonding of enzyme units using bi- or multifunctional reagents, such as GA. It is often applied to form three-dimensional cross-linked enzyme aggregates (CLEAs) that do not require a support. The main

⁸⁴ P. A. Sampaio, E. C. V. Pereira, P. G. S. Sá, J. M. T. Alencar Filho, L. R. M. Ferraz, R. H. V. Nishimura, A. S. Ferreira, P. J. Rolim Neto, E. S. Araújo, L. A. Rolim, *Compounds* 2025, 5(1), 1.

⁸⁵ G. Cirillo, F. P. Nicoletta, M. Curcio, U. G. Spizzirri, N. Picci, F. Iemma, *Reactive and Functional Polymers*, 2014, 83, pp. 62-69.

⁸⁶ X. Liu, Y. Fang, X. Yang, Y. Li, C. Wang, *Chemical Engineering Journal*, 2018, 336, 456-464.

⁸⁷ S. Smith, K. Goodge, M. Delaney, A. Strzyk, N. Tansey, M. Frey, *Nanomaterials*, 2020, 10(11), 2142.

⁸⁸ J. Zdart, K. Jankowska, K. Bachosz, E. Kijeńska-Gawrońska, A. Zgoła-Grześkowiak, E. Kaczorek, T. Jesionowski, *Catalysis Today*, 2020, 348, 127–36.

⁸⁹ S. Pahujani, S. S. Kanwar, G. Chauhan, R. Gupta, *Bioresource Technology*, 2008, 99, 7, 2566-2570.

⁹⁰ Q. Miao, C. Zhang, S. Zhou, L. Meng, L. Huang, Y. Ni, L. Chen, *ACS Omega*, 6, 2021, 47.

disadvantage is that covalent bonds can alter enzyme conformation or involve active site residues, potentially reducing activity.⁹¹ Apart from CLEAs, enzymes can also be immobilized on polymeric supports first and then crosslinked with GA. For example, esterase has been immobilized by adsorption onto polymeric resins and subsequently crosslinked with GA.⁹²

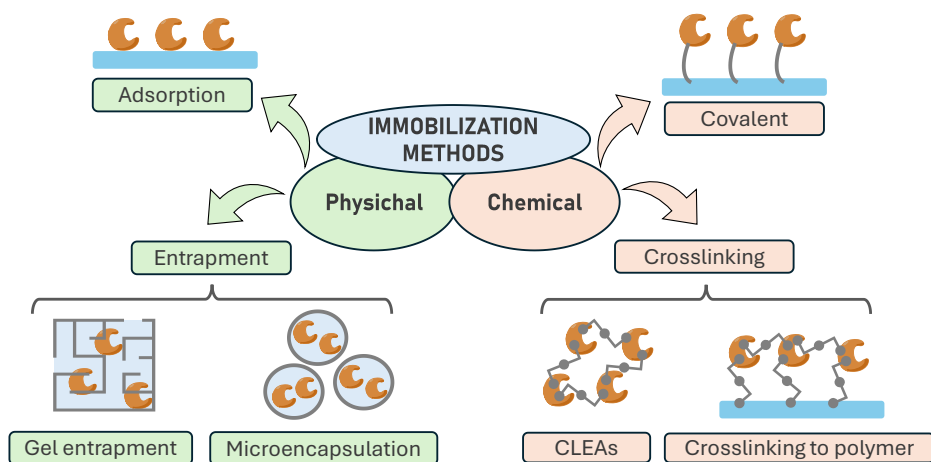


Figure 7. Graphical abstract of the main immobilization methods, classified according to the nature of their interaction with the support.

1.4.2. Thin films as support materials for enzyme immobilization

There is a wide variety of formats in which immobilization polymeric supports can be found, such as fibers, gels, membranes, beads, or thin films, among many others. In this thesis, since we are focusing on thin films, several examples of this format as enzyme supports are presented below, classified according to their chemical nature as either inorganic or organic:

- **Inorganic:** Borosilicate thin films functionalized with 3-aminopropyltriethoxysilane (APTES) have been used to immobilize β -

⁹¹ N. Chen, B. Chang, N. Shi, W. Yan, F. Lu, F. Liu, *Critical Reviews in Biotechnology*, 2022, 43(3), 369-383.

⁹² M. Wang, H. Shi, D. Wu, H. Han, J. Zhang, Z. Xing, S. Wang, Q. Li, *Molecules*, 2014, 19(7), 9838-9849.

glucosidase through electrostatic adsorption.⁹³ Similarly, gold-functionalized surfaces bearing NH_3^+ groups have been applied to immobilize glucose oxidase via electrostatic interactions and β -galactosidase has also been entrapped within siloxane polymer thin films.^{94,95}

- **Organic:** Horseradish peroxidase has been adsorbed on poly(ethylene-co-acrylic acid) thin films.⁹⁶ Poly[1-(2-carboxyethyl)pyrrole] films have been used for the covalent immobilization of glucose oxidase through condensation reactions,⁹⁷ and β -glucosidase has been covalently immobilized on polyethylene-based thin films modified with polystyrene-derived diazonium salts.⁹⁸
- **Hybrid supports:** Synthetic thin films obtained by copolymerizing glycidyl methacrylate, butyl methacrylate, and hydroxyethyl methacrylate on silicon wafers have been employed to covalently immobilize lipases via epoxy groups,⁹⁹ illustrating the potential of hybrid systems for stable enzyme immobilization.

As can be seen, while there are some literature examples of enzyme immobilization in thin films, it is still difficult to find reports on covalent immobilization in dense freestanding organic polymer thin films, which are the support materials proposed in this work. In the following chapters, the publications derived from this thesis will be presented, which exploit these materials for enzyme immobilization.

As previously discussed, these dense thin films offer high structural integrity, homogeneity, and excellent mechanical and chemical stability. In

⁹³ J. Britton, C. L. Raston, and G. A. Weiss, *Chemical Communications*, 2016, 52, 10159–10162.

⁹⁴ G. Ferrand-Drake del Castillo, M. Koenig, M. Müller, K.-J. Eichhorn, M. Stamm, P. Uhlmann, A. Dahlin, *Langmuir*, 2019, 35, 9, 3479–3489.

⁹⁵ A. Elagli, K. Belhacene, C. Vivien, P. Dhulster, R. Froidevaux, P. Supiot, *Journal of Molecular Catalysis B: Enzymatic*, 2014, 110, 77–86.

⁹⁶ X. Su, Y. Zong, R. Richter, W. Knoll, *Journal of Colloid and Interface Science*, 2005, 287, 1, 35–42.

⁹⁷ K. Kojima, T. Yamauchi, M. Shimomura, S. Miyauchi, *Polymer*, 1998, 39, 11, 2079–2082.

⁹⁸ X. Li, X. Wang, G. Ye, W. Xia, X. Wang, *Polymer* 51, 2010, 51, 4, 860–867.

⁹⁹ B. Chen, N. Pernodet, M. H. Rafailovich, A. Bakhtina, R. A. Gross, *Langmuir*, 2008, 24, 23, 13457–13464.

addition, they allow the introduction of specific pendant functional groups on the film surface, which can be used for covalent or non-covalent enzyme attachment. These features make dense thin films an optimal and versatile support for enzyme immobilization, providing stable interactions with enzymes and great reusability, since the material can be easily recovered from the medium due to its robust format.

1.5. Thin films as sensory polymers

As described previously, smart polymers can function as sensory polymers, providing a detectable response (output) when they interact with target molecules (input) through their specific receptors. This response is often optical, manifested as a color change (colorimetric sensors) or fluorescence variation (fluorometric sensors) upon target recognition.¹⁰⁰ Dense thin films have proven to be an excellent format for sensory polymer systems, offering several advantages:

- Exceptional tailorability, which enables the design of custom-made sensors specifically adapted to the target analyte
- High mechanical stability, enabling their direct application in situ.
- A polymeric matrix that creates a favorable microenvironment, enhancing receptor-target interactions.
- Chemical versatility, allowing their use in both aqueous and organic media.
- Ease of handling, making them suitable for use by non-specialized personnel.
- Covalent incorporation of the receptor within the film structure, preventing its leaching into the medium.
- Homogeneous distribution of the colorimetric or fluorescent response across the film surface, ensuring uniform signal generation and reliable quantification

¹⁰⁰ M.Trigo-López, C. Sedano, Foundation of Sensory Polymers, in: *Sensory Polymers: From Their Design to Practical Applications*, Elsevier, 2024, pp. 1–58.

These sensory thin films can translate the chemical interaction between the target and the receptor into a macroscopic optical signal. This signal, manifested as a color or fluorescence change, can be detected by the naked eye. However, for quantitative determination of the target concentration in a sample, laboratory instruments such as UV–Vis or fluorescence spectrophotometers are typically required.

Nevertheless, as an alternative to these expensive and specialized instruments, which often require advanced technical knowledge and complex analysis software, smartphones have recently emerged as a practical and accessible analytical tool. Their widespread availability and user-friendly operation make them suitable for virtually any user. This approach simply involves capturing images of the films using a smartphone camera and subsequently analyzing the color digitally through a mobile application.

1.5.1. Smartphone RGB analysis for sensory thin films

As a rapid and effective alternative to conventional quantification methods for colorimetric and fluorescent thin film sensors, the Polymers Research Group has been working for several years on digital image analysis techniques to quantify target analytes. Several examples of sensory thin films and smartphone-based quantification approaches have been successfully applied in diverse fields, including biomedicine, environmental remediation, the food industry, and document security.¹⁰¹

This color analysis is based on the concept of color spaces. The most commonly used one for this purpose is the RGB space (Red, Green, Blue), although other models such as HSV (Hue, Saturation, Value) and CIELAB (Commission Internationale de l'Éclairage LAB) can also be employed. Each color is mathematically defined by numerical coordinates within these spaces, which can

¹⁰¹ Polymer Research Group, Universidad de Burgos, *Publication list* [online], cited 4 Apr. 2026, available from: <https://publicationslist.org/grupodepolimeros>

be represented geometrically as a cube (RGB), a cone (HSV), or a sphere (CIELAB).¹⁰²

In the RGB color space, each color in a digital image is defined by a combination of three numerical values ranging from 0 to 255, corresponding to the R, G, and B coordinates. Therefore, when photographing a thin film polymer sensor, three values are obtained, representing the RGB coordinates. One or more of these coordinates can be correlated with the target concentration, thus converting a visual perception into a quantitative numerical value that reflects the analyte content in the sample.¹⁰³

The significance of this innovative method has led to the development of a mobile application, named Colorimetric Titration, created by the Polymer Research Group for both Android and iOS platforms. This app extracts RGB and HSV color parameters from captured images and determines which parameters best correlate with the target concentration. Once a calibration curve is established, the app can process data from unknown samples and calculate the analyte concentration by interpolation within the calibration curve (Figure 8).¹⁰⁴

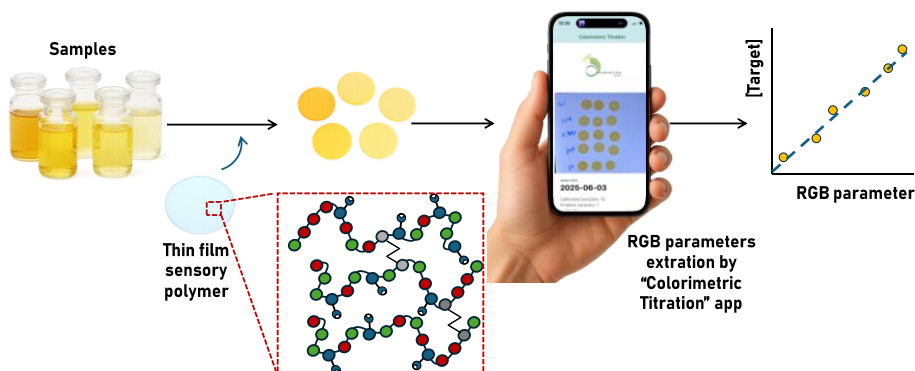


Figure 8. Workflow for smartphone-based RGB analysis using smart sensory films for colorimetric target detection.

¹⁰² Y. Fan, J. Li, Y. Guo, L. Xie, G. Zhang, *Measurement*, 2021, 171, 108829.

¹⁰³ N. Phuangsaijai, J. Jakmunee, S. Kittiwachana, *Journal of Analytical Science and Technology* 2021, 12, 19.

¹⁰⁴ Universidad de Burgos, *Colorimetric Titration* [mobile application], cited 18 Dec. 2025, available from: <https://apps.apple.com/app/colorimetric-titration/id1533793244>

1.6. Research approach. Identified problems and proposed solutions.

As discussed throughout this general introduction, smart polymers, particularly in their thin film format, represent materials with remarkable potential for research and application across multiple sectors. In this thesis, these smart polymers will be applied specifically in two key fields: the food industry and environmental remediation, exploring both of their functional dimensions: as reactive polymers and sensory polymers.

Below, these two areas of application are outlined, along with the proposed smart polymer-based solutions.

Food Industry

With the continuous growth of the global population, it has become essential to increase food production and shelf life, while ensuring food safety and quality. Consequently, it is necessary to develop more efficient and cost-effective production strategies, together with simple and affordable analytical tools for food control and safety. In many food industry processes, enzymes are used to obtain specific products. However, these processes are often inefficient, and since enzymes cannot be reused, they become a bottleneck in production. At the same time, quantifying and evaluating the nutritional composition of food products is crucial, but many existing analytical procedures rely on expensive and complex instrumentation, as well as significant consumption of reagents and organic solvents.

In this context, the present thesis proposes various smart polymer-based thin film systems for application in the food industry, particularly in the dairy and beetroot sector.

On one hand, a reactive thin film with covalently immobilized β -galactosidase on its surface is proposed, allowing continuous operation and enzyme reuse for the production of lactose-free milk. Additionally, a sensory thin

film has been developed, capable of quantifying the protein concentration in whey and related dairy products. On the other hand, two sensory films have also been developed for the quantification of betalains and antioxidant capacity in beetroot.

Environmental remediation

One of the major global challenges is the increasing presence of organic and inorganic pollutants in water sources. Conventional treatment technologies are often costly, energy-intensive, and non-selective and also generate secondary waste. Likewise, analytical methods for pollutant detection, including chromatographic or spectroscopic techniques, require sophisticated equipment, trained personnel, and expensive reagents, which hinders their implementation for on-site monitoring.

To address these limitations, this thesis proposes two smart polymer-based thin films, the first incorporates the enzyme laccase to catalyze the degradation of organic pollutants in aqueous media, while the second is designed for the capture and colorimetric detection of nitrates in water.

1.7. Objectives

This thesis is a multidisciplinary work carried out through the collaboration of the “Polymers” and the “Biochemistry and Biotechnology” Research Groups at the University of Burgos. The general objective of this research is to develop smart polymeric materials in the form of dense thin films, both with and without immobilized enzymes on their surface, for their application as reactive and/or sensory polymers in two key areas: the food industry and environmental remediation. To achieve this objective, the following specific objectives were established:

- To design, develop and characterize smart polymer thin films for applications in the dairy food industry: one as a reactive polymer with immobilized β -galactosidase for the production of lactose-free milk, and
-

another as a sensory polymer to quantify protein content in whey and its derivatives.

- To design, develop and characterize smart polymer thin films for applications in beetroot: one as a sensory polymer for the quantification of betalains, and another as a sensory polymer to determine their antioxidant capacity.
- To design, develop and characterize smart polymer thin films for applications in environmental remediation: one polymer with a dual role, reactive and sensory, to detect and capture nitrates from water, and another reactive polymer with immobilized laccase enzyme for the degradation of bisphenol A and indigo carmine in wastewater.

1.8. Structure of the thesis

The present thesis is divided into three chapters. The first one, the current chapter, provides a general introduction aimed at establishing the context, background, and objectives for the subsequent chapters. In the following chapters, the specific context, methodology, and results are described in detail, organized according to the application area.

Chapter 2 presents the work carried out for the development of smart polymer thin films for applications in the food industry, particularly in the dairy and beetroot sectors. It describes in detail the development of a reactive thin film with covalently anchored β -galactosidase, including the proof-of-concept experiments conducted directly in milk. In addition, within the same application area, a colorimetric sensing polymer is presented for the rapid and simple quantification of protein concentration in whey and related dairy products, using smartphone-based detection. Moreover, this chapter also includes the development of two

sensing polymer films for beetroot, one designed for the quantification of betalains and another for determining antioxidant capacity.

Chapter 3 focuses on the development of smart polymer thin films for environmental remediation, specifically in water treatment. It first describes the development of a reactive thin film with covalently immobilized laccase enzyme, subsequently applied for the degradation of phenolic compounds such as bisphenol A (BPA) and other pollutants like indigo carmine. Finally, the chapter includes the development of a dual-function thin film, acting both as a sensor and reactive material, capable of removing nitrates from water while simultaneously detecting them visually.

It is important to note that the introduction, experimental procedures, materials, results, discussion, and conclusions of each individual study have been published in peer-reviewed scientific journals during the course of this doctoral research, and are therefore reproduced in full within these chapters.

The manuscript concludes with a final chapter of general conclusions, summarizing and integrating all the work carried out throughout this thesis.

CHAPTER 2

Smart polymers in food industry

This chapter focuses on the application of smart polymers in thin-film format within the food industry, with particular emphasis on the dairy and beetroot sectors.

In the dairy field, the first study introduces a thin film bearing pendant NH_2 groups, used as a support for the covalent immobilization of β -galactosidase through azo coupling. This enables the development of a reusable smart polymer capable of producing lactose-free milk by simple immersion. This work was published in *European Polymer Journal* (2023). The second contribution in the dairy sector derives from the same amino-functionalized thin film, here proposed as a sensory smart polymer for quantifying protein concentration in whey and whey protein concentrate samples. The system exhibits a visible color change correlated with protein content due to covalent binding through azo coupling, and the quantification can be easily performed using digital color analysis from photographs taken with a smartphone. This study was published in *Food Chemistry* (2026).

Regarding the beetroot industry, the first study describes a sensory polymer for the colorimetric determination of betalains in real beetroot samples. This polymer incorporates N,N-dimethylaminoethyl methacrylate (NDA) as the receptor monomer, forming positively charged pendant groups capable of interacting with betalain molecules. The betalain concentration can be quantified by analyzing color variation using smartphone photography. This research was published in *Food Chemistry* (2024). The final study presents another sensory polymer for the colorimetric assessment of antioxidant capacity in beetroot samples. This material contains 2,2-diphenyl-1-picrylhydrazyl (DPPH) motifs incorporated via solid-phase synthesis in the thin film, which undergo a characteristic color change when antioxidants alter the oxidative state of DPPH. The intensity of this color change can also be quantified by smartphone-based photographic analysis. This work was also published in *Food Chemistry* (2025).

All four articles are reproduced in full in the following chapter. Each includes its own introduction that contextualizes the respective study, along with its materials, experimental procedures, results, and conclusions.

Film-shaped reusable smart polymer to produce lactose-free milk by simple immersion.

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Film-shaped reusable smart polymer to produce lactose-free milk by simple immersion

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Abstract

In this study, we report the synthesis and characterization of a highly manageable polyacrylic film material for enzyme immobilization, using β -galactosidase (β -gal) as a model enzyme. The material is based on commercially available monomers and achieves efficient immobilization of β -gal through the formation of azo linkages between amino styrene groups in the polyacrylic material and the enzyme. The immobilized enzyme demonstrates superior performance compared to free enzyme in lactose hydrolysis of UHT milk, achieving lactose concentrations below 0.1% (<1 mg/mL), indicating its potential for lactose hydrolysis in dairy products. The film-shaped material is designed for easy submersion and removal, similar to a smart card, and offers reusability, with the ability to be reused at least 10 times without loss of enzymatic activity. Characterization of the immobilized enzyme on the polymeric material was performed using various techniques, including scanning electron microscopy (SEM), Fourier-transform infrared spectroscopy (FT-IR), and Raman spectroscopy. Protein release studies confirmed the stability of the immobilized enzyme during prolonged incubation in aqueous solution without significant enzyme leakage. Overall, the polyacrylic film material demonstrates promise as a simple and efficient approach for enzyme immobilization, with potential applications in various industries, including the food industry.

KEYWORDS: β -galactosidase; lactose-free; low-lactose; lactose hydrolysis; enzyme immobilization

1. Introduction

Over the past decade, lactose-free products have gained a foothold in the food market, and a wide variety of lactose-free products are currently manufactured, including milk, yogurts, cheeses, creams, margarines, butters, and ice creams, among others.^{1–3} To be considered "lactose-free," a product's glucose and galactose disaccharide concentration must be below a certain threshold, as determined, for instance, by the European Food Safety Authority (EFSA).⁴ In industrial settings, lactose-free products are manufactured by adding neutral lactases (β -galactosidases) as an additive or as a processing aid,³ which irreversibly wastes the enzyme and increases production costs. As a result, immobilizing lactases on polymeric supports has become an area of growing interest, particularly in biotechnology and food science, as this technique allows reusability and often enhances enzyme thermo-stability and pH tolerance.^{5,6}

Numerous studies have been published on the immobilization of β -galactosidase (β -gal) onto a wide variety of supports, which reflects the great interest associated with this type of materials.⁷ Some of the most commonly used supports include pectin-based hydrogels,⁶ arabic gum-based hydrogels,⁸ chitosan,^{9,10} sodium alginate gels,^{5,11} carbon nanotube tubular micromotors,¹² ion exchange resin Duolite A568,¹³ polycaprolactone and silk fibroin based nanofiber,¹⁴ natural silk fibers grafted with polyacrylonitrile,¹⁵ nanosilver reduced graphene oxide nanocomposites,¹⁶ polydopamine coated magnetic particles,^{17,18} epoxy resins,^{19,20} aminated PVC,²¹ polystyrene,^{22,23} or modified cellulose acetate.²⁴ However, the reusability of many of these materials has not been demonstrated, and in those that have, enzymatic activity decreases after the first use, disappearing after several cycles of washing/use.⁶

Likewise, various comparative studies have been carried out between free enzymes and immobilized enzymes. Putting aside the fact that when using free

enzymes, they cannot be reused, the truth is that better results are obtained in terms of lactose hydrolysis compared to immobilized enzymes.⁵

Although numerous immobilized enzyme hydrolysis systems have been investigated, only a few have been scaled up, and even fewer have been applied at an industrial or semi-industrial level. The most notable cases include (1) Centrale del Latte in Milan (Italy), which used SNAM Progetti technology by immobilizing the enzyme in cellulose triacetate fibers; (2) Drouin Cooperative Butter Factory in Australia, which relied on technology developed by Sumitomo Chemical (Japan) to immobilize the enzyme in phenol-formaldehyde resin; and (3) Snow Brand in Australia, which developed a rotating column reactor that could be used as a stirred tank reactor and packed bed reactor.²⁵ However, the main disadvantage of all these methods is the loss of efficiency in lactose hydrolysis after several cycles of use/washing, as in many cases, it does not exceed 70-80% of lactose hydrolyzed. In addition, this reuse is often made difficult by the shape/format of the supports, involving filtration processes, etc.

In this work, we describe a highly manageable polyacrylic film material that can be easily submerged and removed like a smart card, and is based on commercially available monomers. Immobilization of β -gal enzyme was achieved through the formation of azo linkages between amino styrene groups present in the polyacrylic material and the enzyme. The novelty of this material lies primarily in the possibility of reusing it at least 10 times without losing any enzymatic activity, simply by dipping the film in the milk, and surpassing the free enzyme's efficiency in the same experimental conditions. The study (summarized in Figure 1) was concluded with a proof-of-concept experiment, in which lactose hydrolysis was performed in UHT milk to achieve lactose-free products, i.e., lactose concentrations below 0.1% (<1 mg/mL). The experiments were conducted at three different temperatures (4, 25, and 55 °C), and the results showed that the immobilized enzyme outperformed the free enzyme in terms of hydrolysis efficiency. These findings demonstrate the material's potential for lactose

hydrolysis in dairy products, even by the end-user, as the procedure is as simple as “dip-wait-remove”.

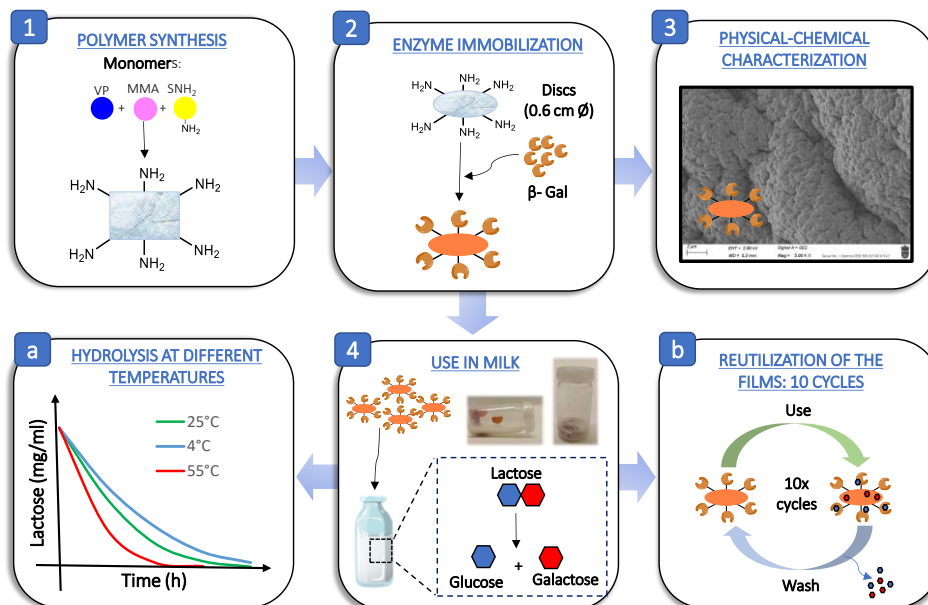


Figure 1. Table of contents of the study.

2. Experimental

2.1. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: 1-vinyl-2-pyrrolidone (VP) (Acros Organic, 99%), methylmethacrylate (MMA) (Merck, 99%) 4-aminostyrene (SNH₂) (TCI, 98%), methanol (VWR-Prolabo, 99.9%), distilled water, hydrochloric acid (VWR, 37%) dimethylsulfoxide-d6 (VWR, 99.8%), citric acid (VWR, ≥99.5%), di-sodium hydrogen phosphate anhydrous (VWR, 99%), sodium carbonate (Sigma-Aldrich, 99.9%), sodium azide (Alfa Aesar, 99%), sodium nitrite (Alfa Aesar, 98%), 2-nitrophenyl β-D-galactopyranoside (ONPG) (TCI, >98%), 2-nitrophenol (ONP) (Acros Organics, 99%), β-galactosidase from *Aspergillus oryzae* (Biolactase F Conc, BIOCON, 45 units.mg⁻¹) (BIO-Beta-gal),²⁶ UHT cow milk (Carrefour). Azo-bis-isobutyronitrile

(AIBN, Aldrich, 98%) was recrystallized twice from methanol. The kit for lactose determination “K-LOLAC” was provided by Megazyme.²⁷

2.2. Instrumentation and methods

The polymers thermal characterization was performed by thermogravimetric analysis (Q50 TGA analyzer, TA Instruments, New Castle, DE, USA) with 10–15 mg of sample under synthetic air and nitrogen atmosphere at 10°C·min⁻¹.

Infrared spectra (FTIR) were recorded with an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) with an ATR-PRO410-S single reflection accessory. RAMAN spectra were recorded with a confocal AFM-RAMAN model Alpha300R – Alpha300A AFM from WITec, using a laser radiation of 785 nm, at magnifications of 100x, 12 mW, 10 accumulations. All spectra were taken at room temperature.

Enzymatic activity assays were performed using a Synergy HT microplate reader (BioTek®, Winooski, Vermont, USA), recording absorbance data at 420 nm. Digital photographs were taken with a smartphone (Mi 9, Xiaomi, Pekín, China)

Field Emission Scanning electron microscopy (FESEM) was carried out using a model GeminiSEM560, ZEISS. Films were dried, freeze fractured, and gold coated in vacuum to ensure the electrical conductivity of the films.

The weight percentage of water taken up by the films upon soaking in pure water at 20 °C until reaching equilibrium (water-swelling percentage, WSP) was obtained from the weight of a dry sample film (w_d) and its water-swelled weight (w_s) using the following expression: $WSP = 100 \times [(w_s \times w_d) / w_d]$.

The protein and fat content of milk was determined following common procedures depicted in the literature, as Bradford and Gerber methods, respectively.^{28,29}

2.3. Design of Beta-gal containing acrylic polymers ($F_{\text{Beta-gal-N3}}$)

From the beginning, we wanted to prepare a manageable material, without needing to handle it delicately or storing it in too careful conditions. In other words, a material to be used by non-specialized personnel in domestic environments, but also in industrial processes where there is no scope to slow down the paces of the industry. In terms of materials science, and regarding the proposed application, this means that the polyacrylic material must contain hydrophobic monomers to provide rigidity, hydrophilic monomers to allow use in aqueous media (such as milk), and aniline groups containing functional monomers for carrying out the immobilization of the enzyme Beta-gal.

For this study, we selected VP (hydrophilic monomer), MMA (hydrophobic monomer), and SNH_2 (functional monomer), using ethylene glycol dimethacrylate (E) as the crosslinking agent.

2.4. Synthesis of Beta-gal containing acrylic polymers ($F_{\text{Beta-gal-N3}}$)

Film-shaped polymers were prepared by bulk radical polymerization of the commercially available monomers VP (45 mol%), MMA (45 mol%), and SNH_2 (10 mol%), the polymeric was crosslinked using 0.1 mol% of E, following the experimental procedure described below.

850 mg (7.64×10^{-3} mol) of VP, 765 mg (7.64×10^{-3} mol) of MMA, 202.5 mg (1.70×10^{-3} mol) of SNH_2 , 3.4 mg (1.70×10^{-5} mol) of E were mixed. Then, 18.2 mg (1.1×10^{-4} mol) of AIBN were dissolved and the solution of comonomers and initiator were injected in a mold ($90 \times 120 \times 0.1$ mm, width, length, thickness) comprised between two silanized glasses in an oxygen-free atmosphere. The polymerization was carried out at 60 °C, overnight, and finally, the films (F_{NH_2}) were washed with water. With the aim of removing unreacted monomers, films were washed with water:acetone mixtures ranging from 100:0 to 0:100, and finally with mixtures 0:100 to 100:0. This gradual solvent transition is performed to avoid breakages associated with sudden changes in the material's WSP.

After the preparation of F_{NH_2} , 3 solid phase reactions were carried out: (1) generation of benzene diazonium salts on the amino groups provided by the SNH_2 monomer ($F_{N_2^+}$), (2) immobilization of the enzyme β -gal through the formation of diazo bonds ($F_{\beta\text{-gal}}$), and (3) quenching of the leftover benzene diazonium groups with sodium azide ($F_{\beta\text{-gal-N}_3}$). The final step is carried out to prevent the highly reactive benzene diazonium groups from reacting with other components of the milk. The schemes of each material are depicted in Figure 2.

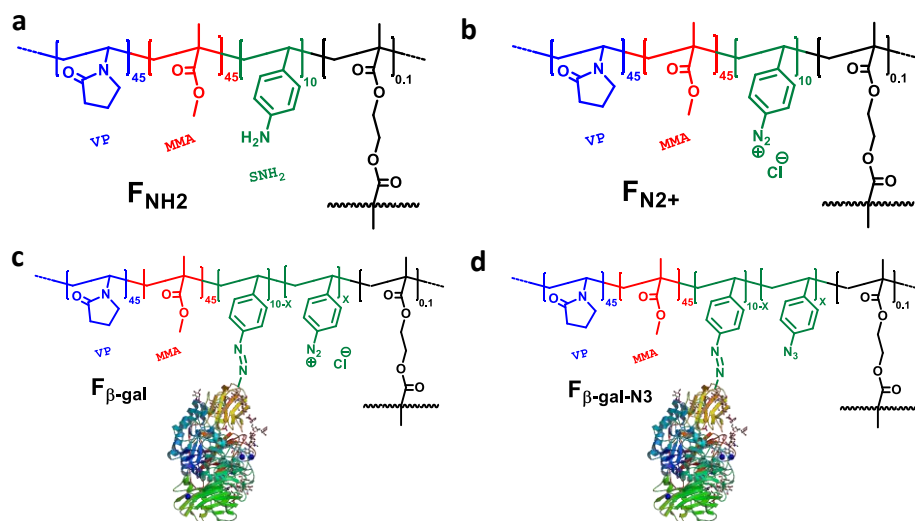


Figure 2. Schematic representation of the prepared materials: a) original film with aminostyrene side groups; b) film with benzene diazonium side groups; c) film with β -gal covalently anchored through azo bonds to the polymer chain, and with remaining benzene diazonium lateral groups; and d) film with β -gal covalently anchored through azo bonds to the polymer chain, and with azidobenzene side groups (enzyme image taken from Wikimedia Commons contributors).³⁰

The first step, namely, the preparation of $F_{N_2^+}$, began with punching F_{NH_2} film into 6 mm diameter discs. Then, 10 disks were dipped for 1 hour in 11 mL of an aqueous solution containing 10 mL of distilled water, 1 mL of HCl (37%), and 50 mg of sodium nitrite. After that, discs were exhaustively washed with distilled water.

The second step was carried out in an Eppendorf vial, by dipping 1 disc of $F_{N_2^+}$ in 1 mL of the aqueous enzyme solution (25 mg of BIO- β -gal per millilitre

of distilled water). The vial was stored overnight at room temperature without stirring, and finally, discs were washed five times with distilled water until no enzyme was detected in the washing water by the Bradford method.²⁸ It is remarkable that the enzyme solution was reused up to 6 times without losing immobilization efficiency. The enzyme quantities, temperature, and pH were optimized with the aim of achieving the highest enzymatic activity possible.

The third step was also performed in an Eppendorf, by dipping 1 disc of $F_{\beta\text{-gal}}$ in 1 mL of aqueous sodium azide solution (5 mg/mL, distilled water). The vial was left at room temperature 1 hour, and without stirring. The $F_{\beta\text{-gal-N}_3}$ discs were washed every 10 minutes five times, and once more overnight.

The complete physicochemical characterization of F_{NH_2} , $F_{\text{N}_2^+}$, $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N}_3}$ can be found in the Supporting Information (SI-Section S1, Figure S1).

2.5. Activity evaluation and protein release study of $F_{\beta\text{-gal-N}_3}$

Enzymatic activity of $F_{\beta\text{-gal-N}_3}$ films were confirmed using ONPG as substrate in a microplate reader with 96-well plates. 6 mm diameter discs were placed at the bottom of each well and 80 μL of a citrate-phosphate pH 5 buffer (di-Sodium hydrogen phosphate 0.2 M and citric acid 0.1 M) and 20 μL of ONPG substrate at a final concentration of 2.5 mM were added. Samples were incubated for 30 minutes at 37 $^{\circ}\text{C}$ and the reaction was stopped by adding 100 μL of sodium carbonate 10% (w/v). The absorbance of the supernatant was measured at 420 nm, and the enzymatic activity was represented as μmol of ONP/h $\cdot\text{cm}^2$ (more information in SI-Section S2, Figure S2a).

Following the same experimental procedure, different experiments were carried out to confirm that the polymeric support was inert, that is, that the material without enzyme (F_{SNH_2} , $F_{\text{N}_2^+}$ and F_{N_3}) did not show activity. In the same way, a film with deposited enzyme (not immobilized, without covalent anchorage) was also tested and labeled as F_{Ads} , which for practical purposes, behaved like a film without enzyme since the successive washes after the deposition process eliminated any enzyme content, making clear the need to carry out a covalent anchorage (more information in SI-Section S2, Figure S2a).

Protein release study was carried out with covalently anchored enzyme-containing films to verify that the enzyme is not liberated from the films in aqueous solution. For that, 10 $F_{\beta\text{-gal-N3}}$ discs (6 mm diameter) were incubated for 30 h in citrate-phosphate pH 6.8 buffer (UHT milk's usual pH). 40 μl of samples were taken at different time points (30 minutes, 1, 6, 20 and 30 h), and total protein content was measured according to the method of Bradford.²⁸

All assays were carried out in triplicate and blanks were used to account for spontaneous breakdown of the substrates (more information in SI-Section S2, Figure S2b).

2.6. Design of the proof of concept

The proof of concept was designed as a comparative test when hydrolyzing commercial milk. Our main objective was to propose a realistic alternative to the current lactose-free milk production method, which is based on adding Beta-galactosidase directly to the milk (Method A). This means that the enzyme is not recovered, and given the boom in lactose-free products in recent years, we believe that a manageable and reusable film-shaped material with low production costs can be postulated as a viable alternative (Method B).

2.6.1. Calibration. Equivalence test.

First of all, it should be pointed out that an attempt was made to determine the amount of the immobilized enzyme by different methods (Bradford²⁸ and Bicinchoninic acid assay³¹), but none of them was satisfactory due to interference of the different methods with the polymeric material. So, since it is a comparative study ("free enzyme" vs $F_{\beta\text{-gal-N3}}$), an experiment was carried out to establish a functional equivalence between the mg of free enzyme used in Method A, and the $F_{\beta\text{-gal-N3}}$ discs used in Method B. To do this, different amounts of BIO- $\beta\text{-gal}$ (0.5, 1, 2.5, and 5 mg) were added to 1 mL of milk. The remaining lactose in each experiment was determined after incubation at 90 rpm for 1 hour at 25 °C, using the kit for lactose determination "K-LOLAC" following the manufacturer instructions. The calibration curve obtained is shown in SI-Section S3 (Figure S3).

Then, the experiment was repeated using 10 discs (6 mm diameter) of $F_{\beta\text{-gal-N3}}$ that were added to 1 mL of milk, and the system was incubated at 90 rpm for 1 hour at 25 °C. The remaining lactose data was introduced in the fitted equation, and the equivalent mg of BIO- β -gal was obtained. Thus, 10 discs of $F_{\beta\text{-gal-N3}}$ are equivalent to 830 μg of BIO- β -gal (they hydrolyze the same amount of lactose, at a given time and temperature).

2.6.2. Comparison. Hydrolyzing lactose from milk with both methods

10 discs (6 mm diameter) of $F_{\beta\text{-gal-N3}}$ were added to 1 mL of milk, and vials were stirred in an orbital stirrer at 90 rpm. The remaining lactose was determined at different times (0, 1, 6, 20, and 30 h) using the kit for lactose determination “K-LOLAC”. The experiment was conducted at three different temperatures (4, 25 and 55 °C). In the same way, the assays were also performed with free enzyme by adding 830 μg of BIO- β -gal to 1 mL of milk.

2.6.3. Storage and reusability

Our design of the material implies a hypothetical future use in the food industry. For this reason, we understand that material reuse is a key point in our development since costs are reduced, and the material complies with the “circular economy” and “environmentally friendly” concepts. Thus, after the first use, the discs were dipped in distilled water for 10 minutes, and the washing process was repeated 5 times as depicted in previous works.³² Then, the films were used again, as described in Section 2.6.2. In total, discs were used/washed up to 10 times.

After the fourth use and washing, discs were stored in the conditions that have turned out to be the most optimal (in the swelled state, packed in zip bags, and at 4°C), and the reusability study was resumed one month later. This proof of concept has been designed to assess the hydrolytic functionality of the material after 10 cycles of use and a storage period.

3. Results and discussion

3.1. Physicochemical characterization of $F_{\beta\text{-gal-N3}}$

The immobilization of β -gal on the polymeric material was characterized by several techniques, being the SEM images shown in Figure 3 the most visual one. The images from F_{NH_2} , and $F_{\text{N}_2^+}$ materials revealed dense structures. Figure 3b shows folds due to the high WSP of the $F_{\text{N}_2^+}$ sample. Unlike F_{NH_2} , $F_{\text{N}_2^+}$ is a polymer that contains 10 mol% of a salt-type co-monomer, which could be understood as a polyelectrolyte with a high water affinity. In fact, F_{NH_2} 's WSP is $30 \pm 1 \%$, while $F_{\text{N}_2^+}$'s is $1,526 \pm 71 \%$. Our interpretation is that the conditions of the SEM analysis (ultra high vacuum) abruptly eliminate the water within the polymeric structure, and wrinkle the material.

Regarding $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N3}}$, Figures 3c and 3d show a rough surface due to enzyme immobilization. Similarly, the cross-section of the materials was also analyzed, but no enzyme was found. That is, immobilization occurs only on the surface, which makes sense due to the size of β -gal (464 KDa), and the dense structures observed before immobilization. The WSP of $F_{\beta\text{-gal}}$ and $F_{\beta\text{-gal-N3}}$ is $516 \pm 26 \%$ and $139 \pm 4 \%$, respectively, since after the immobilization process, there are still active sites (benzene diazonium salt groups) that provide hydrophilicity, which disappear after the reaction of active sites with sodium azide. Therefore, folds can be seen in Figure 3c (still high WSP) but not in Figure 3d.

The presence of enzyme on the surface of the polymeric material was also corroborated by FT-IR and RAMAN, as shown in Figure 4 and SI-Section S1 (Figure S1), respectively.

Materials $F_{\beta\text{-gal}}$ and $F_{\beta\text{-gal-N3}}$ present a wide band at 3300 cm^{-1} due to the N-H vibration band corresponding to the amide bonds of the enzyme. This band is also present in F_{NH_2} , and $F_{\text{N}_2^+}$ materials, but much less intense. In the first case, the two bands are attributed to the asymmetric and symmetric stretching of amine groups (only 10 mol%), while in the second case, the band is unrelated to the chemical structure of the material but rather to the moisture it absorbs. In fact, the

band is a result of water presence in the polymeric material, stemming from its high hydrophilicity. The spectra of the two materials without enzyme (F_{NH_2} and F_{N_2+}) present two vibration bands of $C=O$ at 1721 cm^{-1} and 1674 cm^{-1} , corresponding to MMA and VP, respectively. On the other hand, the materials with enzyme ($F_{\beta\text{-gal}}$ and $F_{\beta\text{-gal-N3}}$) present one extra $\nu_{C=O}$ band, at 1647 cm^{-1} , due to the enzyme's peptidic bonds. It is also noteworthy the presence of the $\nu_{N=N}$ band at 2100 cm^{-1} only in F_{N_2+} and $F_{\beta\text{-gal-N3}}$, due to benzene diazonium salt and azide groups, respectively.

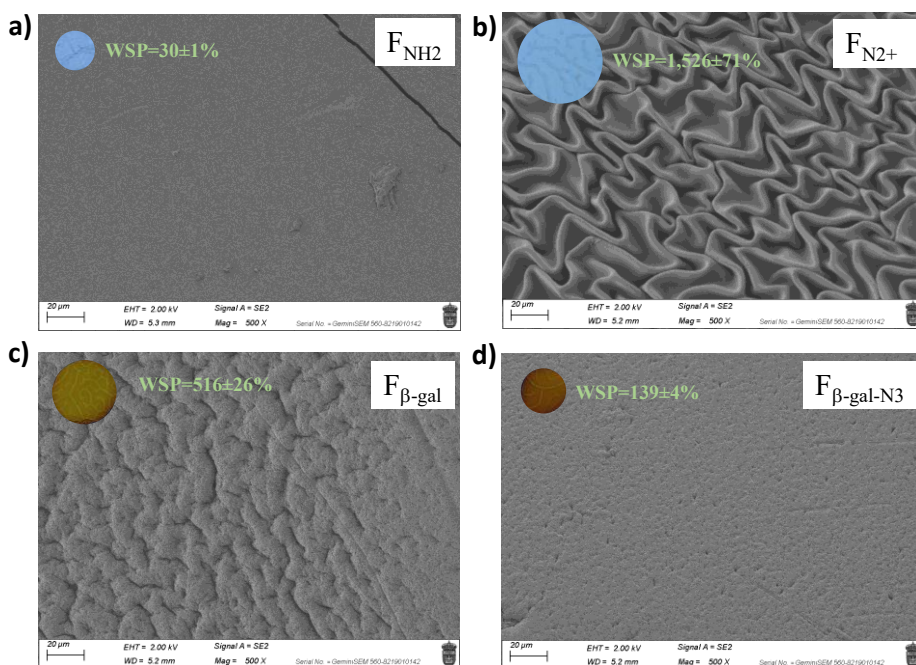


Figure 3. SEM images of the developed material in the different production stages: F_{NH_2} , F_{N_2+} , $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N3}}$. In the upper left corner, the materials' real images (photographs) are shown, visually denoting the WSP and the size differences.

3.2. Evaluation of Beta-galactosidase activity using the acrylic polymer $F_{\beta\text{-gal-N3}}$

The immobilization of the enzyme in the polymeric material does not imply that it has activity. Therefore, in the first stage of validation of the immobilization process, several experiments were conducted. First, we confirmed the Beta-

galactosidase activity of $F_{\beta\text{-gal-N3}}$ disc using ONPG as substrate (SI-Section S2, Figure S2a), verifying that only the film with the enzyme covalently bound ($F_{\beta\text{-gal-N3}}$) presents enzyme activity.

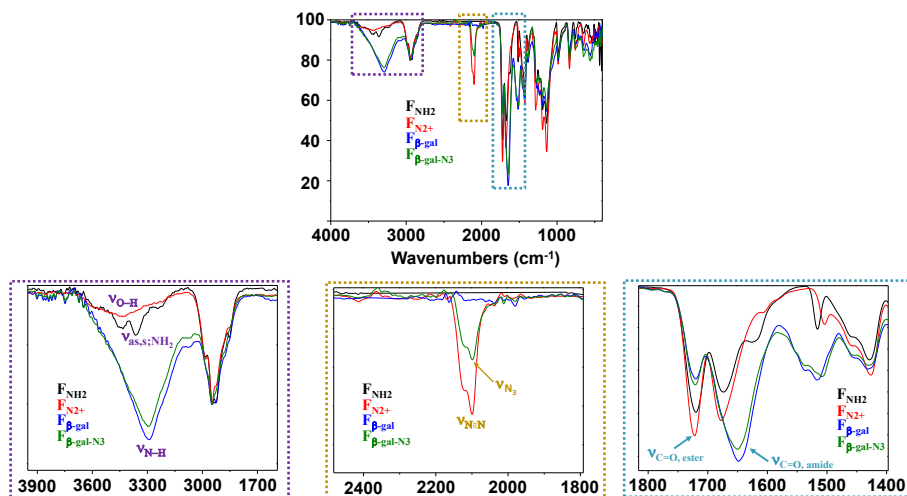


Figure 4. Characterization of polymeric films F_{NH_2} , F_{N_2+} , $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N3}}$ by FTIR.

As these materials are intended for use in the hydrolysis of lactose in milk samples over long periods of time, a protein release study was carried out to validate that the enzyme is not released from the support after long periods in aqueous solution. No protein released to the medium was detected during the 30 h of incubation of the $F_{\beta\text{-gal-N3}}$ discs in citrate-phosphate pH 6.8 buffer (SI-Section S2, Figure S2b), confirming strong binding of the enzyme to the polymeric matrix.

Furthermore, the K_m values for the free and immobilized enzyme were determined (SI-Section S2), and showed no significant differences suggesting that enzyme immobilization did not affect the affinity of the enzyme for the substrate.

3.3. Proof of concept.

The recommended storage temperature for milk is 4°C, so it was one of the temperatures chosen for the study and the most realistic one when considering everyday use. However, two additional temperatures were included to provide a

comprehensive characterization of the material. On the one hand, 25°C was selected as the temperature under normal conditions. On the other hand, the highest temperature was chosen as the optimal working temperature for the enzyme as indicated by the manufacturer (optimal conditions: 55°C and pH=5).²⁶

The results of the experiments using $F_{\text{Beta-gal-N3}}$ were better than using free enzyme in terms of hydrolyzed lactose (or remaining lactose), regardless of the temperature (4, 25 and 55 °C). As shown in Figure 5a, the dashed curves corresponding to the free enzyme are, in all cases, above solid curves corresponding to $F_{\text{Beta-gal-N3}}$. European Food Safety Authority (EFSA) established that the lactose concentration must be below 0.1 g per 100 mL for milk to be considered "lactose-free".⁴ Using $F_{\beta\text{-gal-N3}}$, that objective is met after 20 h at 25 °C, and after 6 h at 55 °C. When the experiment was carried out at 4 °C, a final lactose concentration of 2.5 mg/mL was achieved after 30 h, insufficient to be considered "lactose-free milk", but low enough to be considered "low lactose milk",⁴ which gives rise to future improvements of the material to achieve the objective of lactose concentration <0.1 g per 100 mL, working under refrigerated conditions.

This experiment was also used to verify the nutritional content of milk as described by other authors.¹⁴ The milk was analyzed before and after the hydrolysis process with $F_{\beta\text{-gal-N3}}$ carried out at 4°C for 30 hours, and it was found that there was no variation in either protein content or fat content (more information in SI-Section S4).

In addition, as shown in Figure 5b, the discs were washed/reused 4 times at 25 °C, then stored for 1 month in a zip bag, and finally reused/washed another 6 times at 25 °C, maintaining the same enzymatic activity throughout the process. This point is key to calculating the material's effective costs since although the initial material costs are low, they are reduced even more considering material's reusability (reusable at least 10 times), and the enzyme containing solution's reusability (reusable at least 6 times). In this way, the manufacturing costs for 1 g of $F_{\beta\text{-gal-N3}}$ are estimated in 0.7 euros (1 gram of material is equivalent to 400 discs).

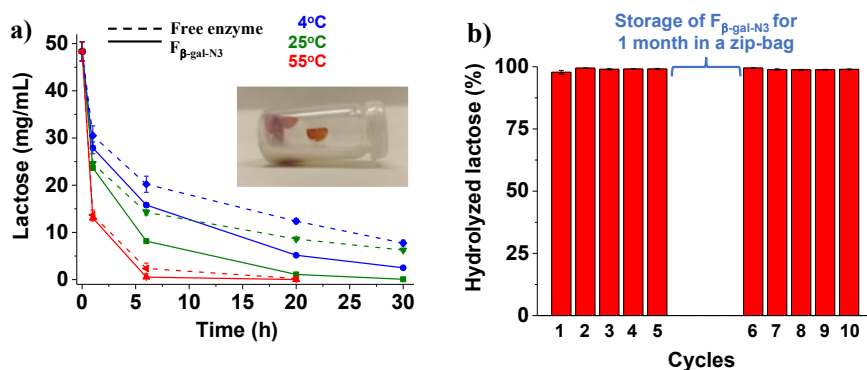


Figure 5. Lactose hydrolysis tests carried out with 1 mL of UHT milk, by adding 830 μ g of BIO-Beta-gal (dashed lines), or with 10 F β -gal-N3 discs (solid lines). a) The graph represents the remaining lactose using both BIO-Beta-gal and F β -gal-N3 discs (inset), and was quantified with the "K-LOLAC" kit (Megazyme) at different times (0, 1, 6, 20 and 30 h) and different temperatures (4, 25 and 55 °C). b) The experiments were carried out at 25 °C, and the hydrolyzed lactose (using F β -gal-N3 discs) was quantified after 30 h with the "K-LOLAC" kit (Megazyme). The films were washed with water and reused ten times following the same experimental procedure, including a one-month storage period after cycle 5. Films were stored in the swelled state, packed in zip bags, and at 4 °C.

3.4. Comparison with other studies

Table 1 summarizes the most relevant papers in the field in recent years, in terms of type of immobilization (covalent/noncovalent), source of the enzyme, whether it has been tested under real conditions (namely, in milk), and whether it is reusable.

Some of the works expose materials with many reuse cycles, but they have not been tested in milk, therefore, it cannot be ensured that other components of the food matrix interfere in the lactose hydrolysis efficiency. Other analyzed materials have been tested in milk, but the efficiency of lactose hydrolyzation in the best case is 82%, and only with 2 use/wash cycles. On the other hand, our material hydrolyzes >99% of lactose after 10 cycles of use/washing in milk, so we consider it a great contribution to the field.

Table 1. Figures of merit: a comparative table of some of the most relevant papers in the field.

Support material	Immobilization (Covalent/ Noncovalent)	Enzyme source	Tested in milk (Y/N)	Cycles of use/wash	Relative activity after last cycle (%)	Hydrolyzed lactose in milk	Incubation Time /Temp (°C)	Ref.
Hydrogels	Noncovalent	<i>Kluyveromyces lactis</i>	Y	6	16	-	20 min / 37	6
Sodium alginate	Noncovalent	<i>Bacillus circulans</i>	N	8	60	-	60 min / 30	5
Arabic-gum hydrogels	Noncovalent	<i>Lactomax 200 s</i>	N	3	94	-	- / 37	8
Carbon nanotubes micromotors	Covalent	<i>Kluyveromyces lactis</i>	Y	2	-	82	20 min / 37	12
Ion exchange duolite A568 + GA	Covalent	<i>Aspergillus Oryzae</i>	N	30	100	-	1 day / RT	13
Polycaprolactone and silk-fibroin based nanofiber fibroin	Noncovalent	-	Y	1	-	41.8	24 h / 4	14
Silk fibers	Covalent	<i>Escherichia coli</i>	N	6	92	-	6 h / 50	15
Nanosilver reduced graphene oxide	Noncovalent	<i>Aspergillus oryzae</i>	N	10	85	-	15 min / 37	16
Polymer coated magnetic particles	Covalent	<i>Aspergillus oryzae</i>	N	20	58	-	5 min / 30	17
Epoxi based polymeric film	Covalent	<i>Escherichia coli</i>	N	12	51	-	15 min / 37	19
PVC+GA	Covalent	<i>Aspergillus oryzae</i>	N	10	98	-	30 min / 40	21
Polystyrene nanofiber	Noncovalent	<i>Kluyveromyces lactis</i>	N	9	30	-	10 min / 37	22
Polystyrene microspheres attached L-alanine	Covalent	<i>Escherichia coli</i>	N	10	73	-	10 min / 30	23
Polyacrylic film F β -gal-N3	Covalent	<i>Aspergillus oryzae</i>	Y	10	>99%	>99%	30 h / 25	This Work

4. Conclusions

In conclusion, we have developed a polyacrylic film material for enzyme immobilization, exemplified by the successful immobilization of β -galactosidase (β -gal) enzyme through the formation of azo linkages. The immobilized enzyme exhibits superior efficiency in lactose hydrolysis in UHT milk compared to the free enzyme, highlighting the promising potential of our polyacrylic film material for application in dairy products. The material was prepared in a film shape for easy submersion and removal, and showed excellent reusability, with the ability to reuse it several times without loss of enzymatic activity. Notably, this polyacrylic film material holds promise for broader applications beyond lactose hydrolysis in dairy products. The general immobilization of enzymes using this material presents potential perspectives in various areas of biocatalysis and biotechnology. Moreover, the potential for tailoring the material properties by modifying the monomer composition or incorporating functional groups could offer new opportunities for fine-tuning the performance of the immobilized enzymes. Additionally, the film shape and reusability of the material make it attractive for continuous and batch processes, providing practical advantages in industrial settings.

Open Data

Open Data is available at <https://riubu.ubu.es/handle/10259/5684> (Dataset of the work "Film-shaped reusable smart polymer to produce lactose-free milk by simple immersion").

Competing interests

The authors declare no competing interests.

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Supplementary Material

Characterization of polymeric films F_{NH_2} , F_{N_2+} , $F_{\beta\text{-gal}}$, and $F_{\beta\text{-gal-N}_3}$ by RAMAN and TGA; calibration curve of the equivalence test; biochemical characterization of $F_{\beta\text{-gal-N}_3}$; determination of the nutritional content of milk.

Authors partially generated the graphical abstract with DALL-E, OpenAI's large-scale image-generation model from natural language descriptions. Upon generating part of the image (the white surface with small balls), the author reviewed, edited, and revised the final image to their own liking and takes ultimate responsibility for its content.

The Supplementary Material is available free of charge at:

<https://ars.els-cdn.com/content/image/1-s2.0-S001430572300678X-mmc1.pdf>

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Dual-range colorimetric protein quantification in whey and WPC using a smart polymeric film and RGB imaging

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Dual-range colorimetric protein quantification in whey and WPC using a smart polymeric film and RGB imaging

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Abstract

Whey is an important dairy by-product whose protein content must be accurately determined for quality control and fraud detection. Conventional nitrogen-based methods (e.g., Kjeldahl) require laboratory equipment and overestimate protein in the presence of non-protein nitrogen compounds. Here, we report a rapid analytical approach based on a smart polymeric film (FRGB) prepared by diazotisation–azo coupling chemistry. The film reacts with aromatic residues of whey proteins, producing a colour change quantifiable from a smartphone image. The sensor displays a dual-range response: the blue channel provides high sensitivity at low concentrations, whereas the green channel remains linear at high concentrations, enabling direct analysis of whey protein concentrate without dilution. The method showed excellent linearity in whey ($R^2 = 0.99$) and commercial WPC over 0–120 mg·mL⁻¹, with a detection limit of 0.284 mg·mL⁻¹ and recovery rate of 94±1.46%. Non-protein nitrogen compounds did not interfere, allowing rapid screening and preliminary authenticity assessment of whey-derived products.

KEYWORDS: Smart Polymers, Films, Whey, WPC, RGB Analysis, Protein Analysis

1. Introduction

Dairy products play a central role in global nutrition. In 2023, worldwide milk production reached approximately 965.7 million tons, of which about 160.8 million tons were produced in Europe (University of Wisconsin–Madison Division of Extension, n.d.). A large fraction of this milk is transformed into cheese, butter or yoghurt, generating whey as the main by-product. Cheese manufacture produces approximately 9–10 L of whey per kilogram of cheese and, due to its high organic load, its disposal represents an environmental concern.^{3,4} Once considered waste, whey is now recognised as a valuable resource because of its high-quality proteins and bioactive components, and its valorisation contributes to sustainability and circular economy strategies.³

Among whey-derived products, Whey Protein Concentrates (WPC) are widely produced through ultrafiltration and diafiltration followed by pasteurisation, evaporation and drying ^{5,6}. WPC typically contains 25–80 % protein (dry basis), with commercial grades such as WPC-35 and WPC-80, whereas Whey Protein Isolates (WPI) contain ≥ 90 % protein according to widely adopted industry standards (ADPI). Because product classification, labelling and commercial value depend directly on the declared protein percentage, accurate protein quantification is essential for regulatory compliance, quality control and detection of economically motivated adulteration and for commercial grading and pricing of whey protein ingredients.⁷

Protein determination in whey and WPC is traditionally performed using nitrogen-based reference methods such as Kjeldahl and Dumas, or spectrophotometric assays including Bradford, Lowry, Biuret and A280 measurements.^{8–11} Kjeldahl and Dumas quantify total nitrogen and convert it into protein content using a fixed nitrogen-to-protein conversion factor, assuming that all nitrogen originates from proteins. Consequently, they cannot distinguish true proteins from non-protein nitrogen compounds.^{12–14} Conversely, chromogenic assays require dilution, controlled conditions and laboratory instrumentation,

which limits their applicability to highly concentrated WPC samples and prevents rapid analysis.

Beyond final product certification, protein concentration must also be monitored during industrial processing. During ultrafiltration and diafiltration, operators need to determine when the target concentration factor has been reached and whether blending or further concentration is required. In practice, reference methods such as Kjeldahl or Dumas are performed off-line in quality control laboratories and results are obtained hours after sampling. As a result, processing decisions are typically based on indirect parameters such as conductivity or refractive index rather than direct protein measurements.

Therefore, a practical analytical gap exists: there is currently no simple, direct chemical method capable of assessing protein concentration in both dilute whey streams and highly concentrated WPC solutions on-site, without dilution or specialised instrumentation.

Despite their widespread use, current protein quantification methods present important limitations when applied to whey protein concentrates (WPC). Nitrogen-based assays such as Kjeldahl quantify total nitrogen rather than other methods, leading to overestimation in the presence of non-protein nitrogen compounds (e.g., urea, ammonium salts, or melamine).^{12–14} In contrast, spectrophotometric and chromogenic assays (Bradford, Lowry, or Biuret) are solution-phase methods with limited linear ranges, typically requiring multiple dilution steps for concentrated WPC, a limitation especially relevant for highly concentrated WPC matrices and one that introduces cumulative errors and reduces practicality for rapid screening.^{8–11}

Although portable and polymer-based sensing strategies have been reported for food analysis, most operate in diluted solutions and within relatively narrow dynamic ranges; therefore, they still require pretreatment or dilution for protein-rich matrices such as WPC and often rely on controlled optical conditions or laboratory equipment. This limitation is particularly relevant in industrial contexts, where rapid decisions must be made by minimally trained personnel. In

parallel, alternative strategies proposed for the authentication of whey protein supplements include mid- and near-infrared spectroscopy and smartphone imaging combined with chemometric or machine-learning analysis.^{13,15–17} These approaches rely on multivariate pattern recognition rather than on a direct chemical interaction with protein functional groups. While effective for classification, they rely on calibration models derived from global spectral or visual fingerprints that may be sensitive to formulation variability and additives, and they do not intrinsically overcome the core limitation of nitrogen-based assays in distinguishing intact proteins from non-protein nitrogen compounds.

Whey protein products remain susceptible to economic adulteration because commercial value is directly linked to declared protein content. When protein is estimated through total nitrogen methods, different adulterants may artificially increase apparent protein content, including free amino acids (e.g., glycine and leucine), urea, ammonium salts, and other nitrogen-rich compounds, while carbohydrate fillers such as maltodextrins may dilute the product. A well-known example is melamine (2,4,6-triamino-1,3,5-triazine), historically associated with major food safety incidents¹⁸. Therefore, analytical approaches that selectively respond to protein-specific chemical structures rather than nitrogen content are desirable for whey protein authentication and fraud screening.

In this context, rapid colorimetric approaches are attractive because of their simplicity, low cost, and suitability for on-site measurements.^{19–21} Our research group has developed polymer-based sensors for food matrices such as beetroots, wines, honey, and processed meats.^{22–28} Building upon this experience, we developed a method to quantify protein concentration in whey and derivatives such as WPC using a smart polymeric film: an acrylic polymer bearing pendant amino groups that can be diazotised in the solid state to generate benzene diazonium functionalities. Upon exposure to whey proteins, covalent attachment via azo coupling produces a distinct colour change (Figure 1).

The objective of the present work is to provide a complementary rapid screening approach for whey-derived products and protein concentrates. The proposed diazonium-functionalised film (F_{RGB}) provides a rapid on-site indication of relative protein concentration based on a single immersion step and smartphone image analysis and is intended as an accessible in-situ control tool for production decisions, raw-material reception, formulation adjustment and authenticity verification. Importantly, it is designed to operate across both dilute whey streams and highly concentrated WPC samples without prior dilution, and to support the detection of dilution or amino-acid-based adulteration.

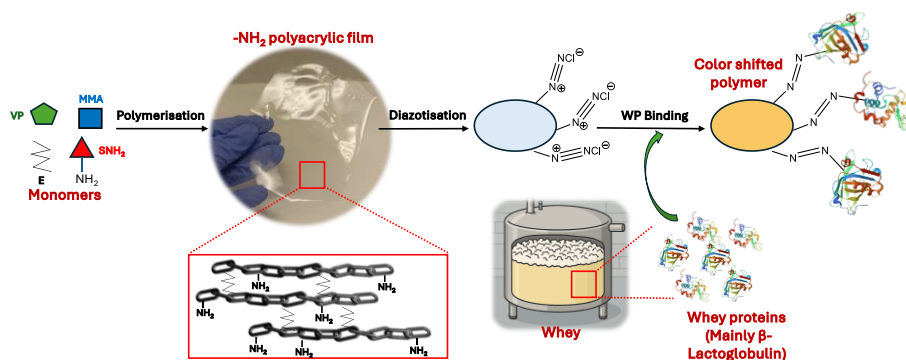


Figure 1. Simplified scheme of film-shaped polymer sensor (F_{RGB}) synthesis and its functionality. First, a polymeric film with pendant NH_2 groups was synthesised by the bulk radical polymerisation of vinylpyrrolidone (VP), methyl methacrylate (MMA), 4-aminostyrene (SNH_2), and the cross-linking agent ethylene glycol dimethacrylate (E). Subsequently, after punching 0.6 cm of diameter disks, a diazotisation reaction was performed, yielding a material containing free benzene diazonium salts. Finally, whey proteins were covalently attached via azo coupling, resulting in a detectable colour change in the film.

2. Experimental

2.1. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: 1-vinyl-2-pyrrolidone (VP) (Acros Organic, 99%), methyl methacrylate (MMA) (Merck, 99%), 4-aminostyrene (SNH₂) (TCI, 98%), urea (TCI, ≥99.0%), melamine (Sigma-Aldrich, 99%), ammonium nitrate (Sigma-Aldrich, ≥99%), methanol (VWR-Prolabo, 99.9%), ethanol (VWR, 99.96%), hydrochloric acid (VWR, 37%), sodium nitrite (Alfa Aesar, 98%), azo-bis-isobutyronitrile (AIBN, Aldrich, 98%), ethylene glycol dimethacrylate (E) (Aldrich, 98%), β-lactoglobulin (β-Lg) from bovine milk (Merck, Approx. 90%), α-Lactalbumin from bovine milk (Merck, Type I, ≥85%), Bio-Rad Protein Assay Dye Reagent Concentrate (BioRad), commercial rennet 1:20000 (Nievi, Productos Nievi, S.A., Spain). Commercial milks: whole milk (Pascual, Calidad Pascual S.A.U, Spain), fresh whole milk (Hacendado, Mercadona S.A., Spain). Whey protein supplements: Evowhey Protein 2.0, coffee with milk flavor (HSN®, HSNstore S.L., Spain), Evowhey Protein 2.0, biscuit flavor (HSN®, HSNstore S.L., Spain)

2.2. Instrumentation and methods

The water-swelling percentage (WSP) of the films, which represents the amount of water absorbed when immersed in pure water at 20 °C until swelling equilibrium was reached, was calculated using the dry film weight (w_d) and swollen weight (w_s) using the following formula: $WSP = 100 \times [(w_s - w_d) / w_d]$.

The thermal properties of the polymers were examined using thermogravimetric analysis (TGA) with a Q50 TGA analyser (TA Instruments, New Castle, DE, USA). Samples of 10–15 mg were tested under synthetic air and nitrogen atmospheres at a heating rate of 10 °C/min.

Differential scanning calorimetry (DSC) measurements were performed using a TA Instruments Q200 DSC analyser under a nitrogen atmosphere at a heating rate of 20 °C/min. The sample sizes ranged from 10 to 15 mg. Data

collection began with a sampling interval of 0.10 seconds per point. Initially, the sample was stabilised at -80.00 °C before starting the first cycle of the experiment. The temperature was then increased to 10.00 °C/min until 150.00 °C, marking the end of the second cycle of the experiment. Cooling down to -80.00 °C followed at the same rate, completing the third cycle of the experiment. Finally, the temperature was increased to 350.00 °C at the same heating rate, completing the fourth cycle, which was used for the analysis of the results.

Fourier-transform infrared (ATR-FTIR) spectra were acquired using an FT/IR-4200 spectrometer (Jasco, Tokyo, Japan) equipped with a single-reflection ATR-PRO410-S accessory.

The tensile properties were evaluated using samples measuring 5 mm × 9.44 mm × 0.103 mm, tested at a rate of 5 mm/min using an EZ Test Compact Table-Top Universal Tester (Shimadzu, Kyoto, Japan).

The films were polymerised between two parallel silanised glass plates separated by a calibrated 100 µm spacer, producing a uniform sheet (~7 × 7 cm). Mechanical specimens were cut from different regions of the same sheet. Thickness was measured at multiple positions using a digital micrometer, yielding an average value of 0.103 mm with minimal variation, confirming the mechanical integrity of the sensing films.

As reference protein quantification methods, protein absorbance at 280 nm (A₂₈₀) was measured using a UV/Vis spectrophotometer (Hewlett Packard 8453) operated with HP ChemStation software. Measurements were performed using quartz cuvettes with a path length of 0.5 mm. The absorbance spectra of the films were recorded using a UV–Vis spectrophotometer (Hewlett Packard 8453) equipped with HP ChemStation software.

The Bradford assay, used as a comparative reference method, was performed using the Bio-Rad Protein Assay Dye Reagent, which was diluted 1:4 with distilled water. A total of 200 µL of diluted reagent and 10 µL of sample were

added to each well of a 96-well microplate. Absorbance at 595 nm was measured using a PowerWave XS2 microplate reader (BioTek).

The limits of detection (LOD) and quantification (LOQ) for the three protein quantification methods (using β -Lg) were calculated using the following standard equations: $\text{LOD} = 3.3 \times \text{SD}/s$ and $\text{LOQ} = 10 \times \text{SD}/s$, where SD represents the standard deviation of the blank measurements and s is the slope of the corresponding analytical calibration curve.

Protein quantification using the F_{RGB} sensor was carried out by immersing a fully diazotised polymer disc in the protein solution for a fixed incubation time under ambient laboratory conditions. After incubation, the discs were rinsed with distilled water to remove unbound material and dried at room temperature. Images of the films were acquired using a smartphone camera under constant lighting conditions and analysed using RGB colour extraction software. The analytical signal corresponded to the variation in the selected RGB channel intensity relative to a blank disc. The blank consisted of fully diazotised F_{RGB} discs subjected to the complete analytical protocol (washing, incubation time, image acquisition and RGB analysis) but incubated in distilled water instead of protein solution. Consequently, the calculated LOD reflects the intrinsic analytical variability of the activated sensing material and the image-analysis procedure rather than the optical background of a non-reacted polymer.

All calibration measurements were performed in triplicate and expressed as mean $\pm$ standard deviation.

2.3. Preparation of acid and sweet whey

Whey obtained by acid precipitation (acid whey) was prepared by skimming whole milk via centrifugation (8000 rpm, 20 min, 4 °C). The resulting skimmed milk was acidified to pH 4.6 using 2 N HCl to precipitate caseins, which were subsequently removed by centrifugation (1800 rpm, 15 min, 20 °C). Whey obtained by enzymatic precipitation (sweet whey) was prepared by adding 0.25 mL of commercial rennet to 500 mL of fresh whole milk that had been previously

heated to 50 °C. The mixture was left undisturbed for 3 hours to allow curd formation. The curd was then broken to extract more whey and left to rest for 15 minutes. Finally, the sample was centrifuged at 4000 rpm for 15 minutes at 4 °C. No additional fat separation step was applied, as the residual lipid fraction remaining after curd removal was minimal and the sensing mechanism targets soluble whey proteins.

The preparation temperatures correspond to standard procedures for acid and rennet whey production. Although mild heating during rennet coagulation may induce limited conformational relaxation of whey proteins, they remain soluble and chemically intact. Because the whey preparation conditions do not induce structural modifications in the proteins that affect the accessibility of their aromatic residues, the interaction with the FRGB polymer remains unchanged and no analytical bias is introduced in the measurements.

2.4. Design and synthesis of film-shaped polymer sensor (F_{RGB})

Three different formulations of the sensory film were prepared, maintaining the structural monomers VP and MMA while varying the crosslinker (E) molar percentage (0.1 and 1 mol%) and the sensor monomer SNH_2 molar percentage (1 and 10 mol%). These compositional variations were designed to achieve an optimal balance between mechanical properties for adequate handling (both in the dry state and after swelling), a strong colorimetric response, and swelling percentage to facilitate proper interaction between proteins and the polymer. Specifically, the three formulations used to prepare 1.8 g films were: (i) 49.5/49.5/1 in molar proportion of VP (926 mg, 8.33 mmol), MMA (833 mg, 8.33 mmol), and SNH_2 (20 mg, 0.17 mmol), respectively, with 0.1 mol% of E (3.3 mg, 16.6 mmol); (ii) 45/45/10 in molar proportion of VP (832 mg, 7.5 mmol), MMA (750 mg, 7.5 mmol) and SNH_2 (198 mg, 1.7 mmol) respectively, with 0.1 mol% of E (3.3 mg, 16.6 mmol); and (iii) 45/45/10 in molar proportion of VP (818 mg, 7.36 mmol), MMA (736 mg, 7.36 mmol) and SNH_2 (195 mg, 1.6 mmol), respectively, with 1 mol% of E (32.4 mg, 0.16 mmol). The synthesis of these polymers was performed via bulk radical polymerisation, using AIBN (17.8 mg, 0.11 mmol) as a

thermal initiator at 60 °C overnight, as described in previous works.^{29,30} The solid films were demoulded and subjected to sequential solvent exchange to remove unreacted monomers and condition them for protein measurement. It was first immersed in distilled water for 15 minutes, then gradually transitioned to 100% acetone, and subsequently back to distilled water. This gradual exchange prevents abrupt swelling changes, which could lead to film rupture.

Once the film was synthesised, a solid-phase reaction was performed. First, 0.6 cm discs were punched from the film and diazotised through the -NH_2 groups provided by the SNH_2 monomer, leading to the formation of benzene diazonium salts and rendering F_{RGB} . For the diazotisation, ten 0.6 cm discs were immersed in 10 mL of distilled water containing 1 mL of concentrated HCl (37%) and 50 mg of sodium nitrite. The reaction was carried out at room temperature without stirring, with variable reaction times ranging from 5 to 60 minutes. After diazotisation, the films were washed with water three times to remove excess reagents and by-products. The resulting diazotised discs were then used in the subsequent protein incubation experiments.

2.5. Protein measurement procedure using the F_{RGB} method

The reaction between diazonium groups in the polymer and some nucleophilic amino acid residues of proteins (His, Tyr), induces a detectable colour change in the polymer matrix, which serves as the analytical signal. The same measurement protocol was applied to all tested protein systems: β -Lg standard solutions, whey obtained from acid and enzymatic milk precipitation, and commercial WPC. Each 0.6 cm diazotised F_{RGB} disc was immersed in 1 mL of protein solution and incubated statically at room temperature (22 ± 2 °C) for 15 min.

After incubation, the discs were photographed using an iPhone 16 inside a backlit lightbox to ensure consistent lighting conditions and improve reproducibility. The closed light box minimises external illumination variability and standardises image acquisition. The analytical signal is obtained from calibration curves generated under fixed acquisition conditions rather than from absolute

RGB values. Therefore, similarly to spectrophotometric methods, different devices would require their own calibration but would not affect the analytical principle of the method.

Protein quantification was performed using calibration curves obtained under fixed acquisition conditions (same device, illumination and geometry). The smartphone–lightbox system therefore acts as a controlled optical reader, analogous to a spectrophotometric instrument operating under fixed settings. Quantification is based on relative variations in colour intensity rather than on absolute RGB values; consequently, each device–illumination configuration requires independent calibration, while the analytical principle remains unchanged.

Colour analysis was performed using the Colorimetric Titration app,²⁶ which extracts mean pixel intensity values from defined regions of interest (see SI-VIDEO). Colour parameters were extracted from two digital colour spaces: RGB (commonly used in electronic imaging) and HSV colour spaces. Additionally, two combinations of RGB values were evaluated, resulting in a total of eight digital colour variables analysed.²⁸ The analytical response was selected objectively: among the evaluated colour variables, the parameter showing the highest calibration linearity and the lowest residual error was chosen as the quantitative signal.

2.6. Optimisation of diazotisation time and pH on the sensor response

To optimise the diazotisation time and sample pH on the polymer's response to protein was evaluated. Discs with a diameter of 0.6 cm underwent diazotisation as described in Section 2.4 for varying durations (5, 15, 30, and 60 minutes), followed by three washing steps, and a 15-minute incubation as described in Section 2.5 with acid whey at different pH values (4.6, 6.0, and 8.0)

2.7. Selectivity and non-protein nitrogen interference study

Selectivity of the FRGB colorimetric response was evaluated by analysing the effect of non-protein components commonly present in whey systems and in

economically adulterated protein products. Diazotised FRGB discs were used for β -lactoglobulin (β -Lg) determination in the presence of lactose (50 mg mL^{-1}).

Additionally, sweet whey samples were supplemented with representative non-protein nitrogen (NPN) compounds. Free amino acids (glycine, leucine and glutamine, 2 mg mL^{-1} each), urea and an ammonium salt were added while maintaining constant pH conditions. The concentrations were selected to simulate adulteration scenarios in which nitrogen content is artificially increased without a corresponding increase in true protein concentration.

All samples were analysed following the FRGB analytical procedure described in Section 2.5, and the colour response was compared with that obtained for non-adulterated whey samples.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 9). Normality of the data distribution was evaluated using the Shapiro–Wilk test and homogeneity of variances using Levene’s test.

Differences among experimental conditions (pH, diazotisation time and interference/adulteration assays) were analysed using one-way ANOVA followed by Tukey’s multiple comparison test ($p < 0.05$). Pairwise comparisons were evaluated using Student’s t-test ($p < 0.05$).

Calibration curves were obtained by linear regression analysis, and correlation coefficients (R^2) were calculated. Method comparison between FRGB and A280 measurements was evaluated by regression analysis. Precision was expressed as relative standard deviation (RSD), and accuracy was assessed by spike–recovery experiments.

3. Results and discussion

3.1 Selection of film formulation and physicochemical characterization

The material was designed as the solid-phase substrate of a rapid screening analytical method rather than as a laboratory reference assay; therefore, ease of

handling, short analysis time and compatibility with concentrated protein matrices were prioritised. The film format was selected because it enables direct immersion measurements without filtration or extraction steps^{25,28}. A balance between mechanical strength, colour response and controlled swelling was required to ensure effective contact between the polymer and whey proteins, allowing the diazotised film to react with aromatic amino acid residues of proteins, predominantly tyrosine, to form the coloured azo linkage.³⁰

Polymer composition was selected to control hydration, protein diffusion and accessibility of reactive diazonium groups. Vinylpyrrolidone (VP) provides a hydrophilic environment that promotes protein diffusion, methyl methacrylate (MMA) confers dimensional stability and limits excessive swelling, and aminostyrene (SNH₂) supplies the aromatic amine groups that are diazotised to generate the reactive sites responsible for azo coupling with protein aromatic residues. Consequently, sensing performance depends on the balance between hydration, mechanical stability and accessibility of the polymer–protein interface.

The F_{RGB} synthesis and protein interaction follow our previous procedure,³⁰ with two modifications: omission of the sodium azide blocking step and reduction of protein incubation time to 15 min, simplifying the assay and shortening analysis time. To select the optimal formulation, three compositions were evaluated using acid whey adjusted to pH 8 (Section 2.5). A previously reported formulation (45/45/10 VP/MMA/SNH₂ with 0.1 mol% crosslinker E),^{29,30} tested here as Material 2 (Table 1), showed excessive swelling (83%) and poor dimensional stability, compromising handling and measurement reproducibility; therefore, it was discarded.

This behaviour represents a physicochemical limitation of the analytical sensing mechanism rather than a simple handling issue. Excessive swelling disrupts dimensional stability and generates uncontrolled mass transport within the polymer network, so the diffusion path length and local protein concentration at the interface vary between measurements, leading to poor signal

reproducibility. This reflects the typical trade-off in solid-phase polymer sensors between functional group density and mass-transport control.

Two alternative formulations were evaluated (Table 1). Material 1 (1% SNH₂, 0.1% E) showed low swelling (38%) and good mechanical stability but produced weak colour signals due to insufficient protein binding. In contrast, Material 3 (10% SNH₂, 1% E) provided strong colour response with moderate swelling (58%), representing an appropriate balance between sensitivity and mechanical stability. Higher SNH₂ contents caused excessive swelling after diazotisation and washing, whereas higher crosslinker contents hindered protein diffusion toward reactive diazonium groups. Diazotisation introduces fixed ionic charges into the network, producing strong osmotic swelling; previous studies reported water swelling above 1500%, leading to poor dimensional stability and preventing reproducible measurements.²⁹ Therefore, these conditions limit analytical applicability rather than merely affecting handling.

Material 3 therefore represents a compromise between reactive group density, mechanical stability and diffusion accessibility rather than simply the highest monomer content. The sensing process is limited by protein diffusion and accessibility of reactive sites, not by the total number of functional groups.

From an analytical standpoint, this formulation maximizes signal-to-noise ratio: increasing reactive group density would increase colour intensity but also swelling-induced variability and diffusion heterogeneity, reducing quantitative reliability. Consequently, the final composition was selected based on calibration linearity and measurement reproducibility rather than colour intensity alone.

Based on these results, Material 3 (10% SNH₂, 1% E) was selected as the optimal formulation for protein detection. Mechanical testing showed adequate flexibility and strength (Young's modulus $1364 \pm 136 \text{ N mm}^{-2}$), ensuring reliable handling during analysis.

TGA indicated sufficient stability ($T_5 = 314.62 \text{ }^\circ\text{C}$; $T_{10} = 331.59 \text{ }^\circ\text{C}$) for drying, storage and routine use. DSC revealed a glass transition temperature (T_g)

of 162.70 °C, confirming that the polymer remains rigid under operating conditions. This is relevant because the F_{RGB} film acts as a solid-phase analytical substrate immersed in aqueous solutions; a T_g well above room temperature prevents deformation upon hydration and supports reproducible protein diffusion and colour response.

Table 1. Composition of the three tested F_{RGB} formulations. Material 1: 49.5 VP (vinylpyrrolidone) / 49.5 MMA (methyl methacrylate) / 1 SNH_2 (4-aminostyrene) // 0.1 E (ethylene glycol dimethacrylate) mol%; Material 2: 45 VP / 45 MMA / 10 SNH_2 // 0.1 E mol%; Material 3: 45 VP / 45 MMA / 10 SNH_2 // 1 E mol%. Last column shows representative images of the F_{RGB} films from the three formulations after 15 min incubation with acid whey adjusted to pH 8 with NaOH 0.1M, keeping the same relative size proportions as in the original samples.

Material	% SNH_2	%E	WSP	Real Image
1	1	0.1	38	
2	10	0.1	83	
3	10	1	58	

ATR-FTIR spectra of the film after protein exposure (Figure 2a) showed clear modifications compared with the diazotised film. A new band at $\sim 1540\text{ cm}^{-1}$ (amide II) and broadening in the $1640\text{--}1660\text{ cm}^{-1}$ region (amide I contribution) indicate incorporation of protein into the polymer matrix. These spectral changes confirm that the colour response arises from covalent azo coupling rather than reversible surface adsorption.

3.2 Optimisation of diazotisation time and pH on the sensor response

At pH 4.6 the colour response was negligible and comparable to the control (Figure 2b). A weak response was observed at pH 6, whereas at pH 8 the signal was clearly developed. No significant differences were detected among diazotisation times (5–60 min), indicating that diazonium formation occurs rapidly

and that extending the reaction time does not influence subsequent azo coupling or colour development.^{31,32}

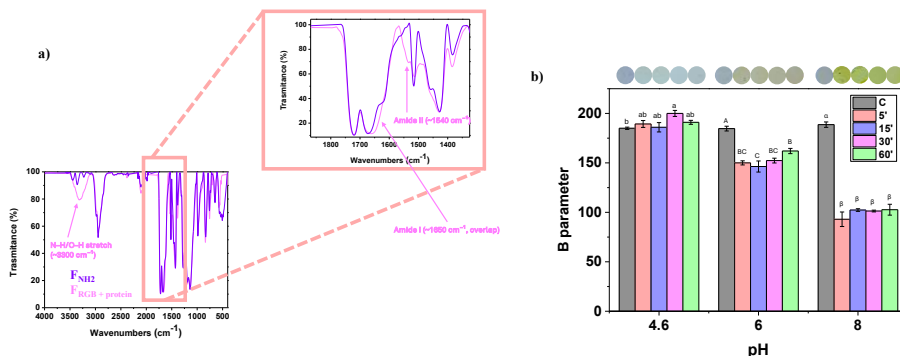


Figure 2. a) ATR-FTIR spectra showing protein incorporation. After incubation, the film exhibits the appearance of an amide II band ($\sim 1540\text{ cm}^{-1}$) and a band broadening in the $1640\text{--}1660\text{ cm}^{-1}$ region associated with the amide I contribution, partially overlapped with the vinylpyrrolidone carbonyl vibration. These spectral changes confirm protein incorporation into the polymer matrix. b) Diazotisation time and pH optimisation. The Y-axis represents the B parameter of the F_{RGB} disks diazotised for different times (5, 15, 30, and 60 min) and subsequently incubated with acid whey adjusted to three different pH values (4.6, 6, and 8) using 0.1 M NaOH. C corresponds to the control films incubated with distilled water adjusted to each pH. Real film images at each time and pH are shown. Bars represent the mean of three replicates $\pm$ SE. Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test. Different letters above the bars indicate statistically significant differences among treatments within the same pH group ($p < 0.05$).

The pH dependence can be attributed to the reactivity of aromatic residues of proteins toward diazonium groups. At acidic pH these residues are protonated and poorly reactive, whereas under mildly alkaline conditions their nucleophilicity increases, favouring azo bond formation.^{33–35}

In particular, tyrosine residues become increasingly reactive under mildly basic conditions (pH 6–8), promoting azo coupling with the diazonium groups.^{36–}

38

In addition to acid–base effects, mildly alkaline conditions may slightly relax whey protein tertiary structure (e.g., β -lactoglobulin), increasing solvent exposure of aromatic residues and their accessibility to the diazonium-

functionalised surface. The higher signal at pH 8 therefore reflects both increased nucleophilicity and improved accessibility of reactive sites.

At higher pH values, aromatic diazonium groups become less stable in aqueous media and may undergo hydrolysis, reducing the effective density of reactive sites. Thus, although alkaline conditions increase reaction rate, they compromise reproducibility. pH 8 therefore represents a compromise between protein nucleophilicity and diazonium stability, providing robust sensor performance.

Mass transport within the polymer matrix facilitates the reaction but does not appear to be the dominant factor governing the signal. Diazotisation introduces ionic groups that hydrate the network and allow diffusion of soluble whey proteins toward the reactive sites. The higher response observed at pH 8 is therefore mainly attributed to the increased nucleophilicity of aromatic residues and faster azo coupling. Because calibration and sample measurements were performed under identical pH conditions, these effects do not bias quantification but rather improve reaction efficiency and reproducibility.

3.3 Linearity calibration with β -Lg using F_{RGB} , Bradford, and A280 methods

β -Lg is the predominant protein in whey, representing approximately 55–65% of its total protein content and its concentration is about 3–4 mg mL⁻¹.^{6,39,40} The isolated protein was used to validate the protein-specific response of the sensor. This validation supports the use of the method for analysing whey and concentrated whey protein samples, ensuring that the sensor response is due to protein interactions. β -Lg was diluted in Milli-Q water to concentrations ranging from 1 to 8 mg mL⁻¹ for the A280 and F_{RGB} methods, and from 0 to 1.2 mg mL⁻¹ for the Bradford method due to its limited linearity above this concentration. Dilutions were then measured using the Bradford protocol, A280, and F_{RGB} method.

As expected, a linear trend was observed in the calibration curves of β -Lg measured by A280 and the Bradford assay (A595) (Figure 3a and 3b,

respectively), both of which are widely established methods for protein quantification.^{8,10,41} Notably, the RGB-based method also showed a strong linear correlation with protein concentration, reaching an R^2 value of 0.99 for the B parameter (Figure 3c). This behaviour can be attributed to the increasing formation of azo bonds between the diazotised polymer and aromatic amino acids (primarily tyrosine and histidine) present in the protein structure.⁴² As the protein concentration increases, more reactive sites are available for the coupling reaction, leading to a more intense chromogenic response on the polymer surface, which is then effectively captured by digital colour analysis.^{30,43}

As shown in Figure 3d, the potential interference of lactose was evaluated, given that it is one of the major components of whey, typically present at concentrations of approximately 50 mg mL⁻¹.⁴⁴ As illustrated, the B parameter remained unchanged in the presence of lactose, indicating that even at high concentrations, lactose does not affect the colourimetric response of the sensor. Therefore, it can be considered that lactose does not interfere with protein quantification in complex matrices such as whey or commercial whey protein preparations.

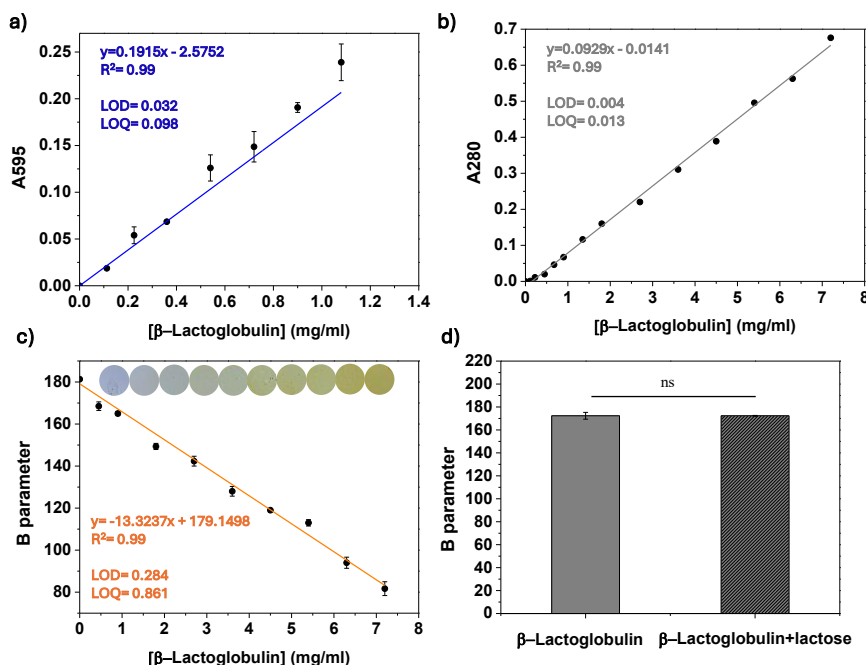


Figure 3. a) Calibration line of β -lactoglobulin (β -Lg) (mg mL⁻¹) using the Bradford method. 200 μ L Bio-Rad Protein Dye diluted 1:4 with 10 μ L sample. b) Calibration line of β -Lg (mg mL⁻¹) using the A280 method with 0.5 mm path-length quartz cuvettes. c) Calibration line of β -Lg (mg mL⁻¹) using the FRGB method; the Y-axis represents the B parameter, and real film images at each concentration are shown. d) Interference assay of the FRGB method with lactose (50 mg mL⁻¹). The Limit of Detection (LOD) and Limit of Quantification (LOQ) values for each method are also displayed. Error bars represent the standard deviation of triplicate measurements ($n = 3$). In the A280 panel, the experimental variability is smaller than the marker size and therefore the error bars are not visually distinguishable. Statistical significance was assessed using Student's t-test ($p < 0.05$). "ns" denotes non-significant differences.

3.4. Linearity in whey samples and interference study.

After confirming linearity with β -lactoglobulin (β -Lg), the method was evaluated in a complex matrix using sweet whey (Section 2.3). The protein content of the whey was first determined by the Bradford assay and dilutions (0–5 mg mL⁻¹) were analysed following the FRGB procedure. A strong correlation between the B parameter and protein concentration was obtained ($R^2 = 0.99$; Figure 4a), similar to that observed with β -Lg. The response is mainly governed by β -Lg, the most abundant whey protein containing accessible aromatic residues,^{6,45} although

other whey proteins may also contribute through azo coupling. Additional structural and experimental evidence supporting the contribution of different whey proteins to the sensor response is provided in the Supplementary Information (SM-Section S2, Figure S2). Because the reaction targets protein-bound aromatic residues, the method provides an estimate of total protein concentration rather than protein structure or conformation. As in other colorimetric protein assays, differences in individual protein response factors are averaged through calibration with a representative whey protein, enabling reliable estimation of overall protein concentration.^{3,46,47}

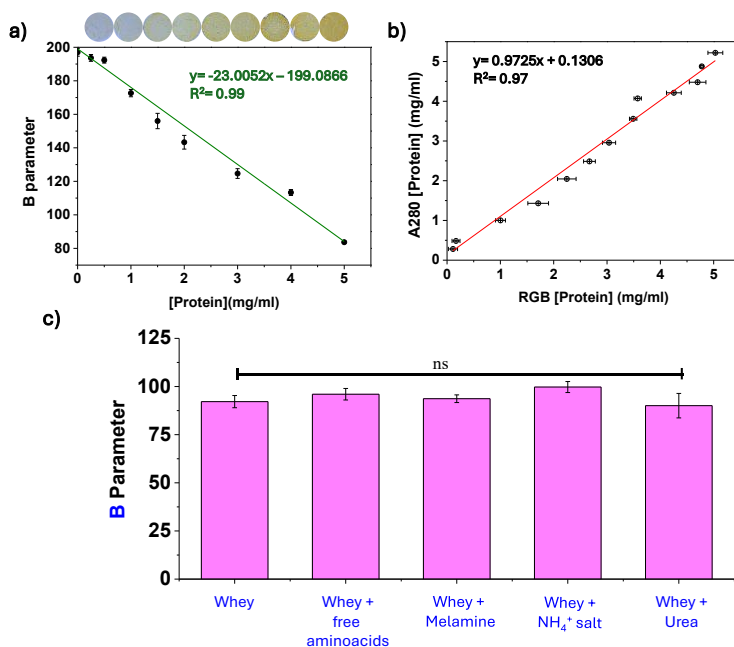


Figure 4. a) Calibration curve of the F_{RGB} method using sweet whey. Representative film images after incubation at each protein concentration (mg mL^{-1}) are shown. b) Comparison of protein concentration (mg mL^{-1}) in whey determined by the F_{RGB} and A280 methods. c) Adulteration scenario: effect of non-protein nitrogen (NPN) compounds on the FRGB response. Sweet whey (5 mg mL^{-1} protein) was supplemented with free amino acids (glycine, leucine and glutamine, 2 mg mL^{-1} each; total 6 mg mL^{-1}), urea (5 mg mL^{-1}), ammonium salt (5 mg mL^{-1}) and melamine (5 mg mL^{-1}). No significant variation in the B parameter was observed, demonstrating selectivity of the sensing mechanism toward protein-bound aromatic residues. Bars represent mean values $\pm$ SE ($n = 3$); "ns" indicates non-significant differences (Student's t-test, $p > 0.05$).

Although β -lactoglobulin dominates the response, the sensing mechanism is not protein-specific. Other whey proteins such as α -lactalbumin and bovine serum albumin also undergo azo coupling, with lower response factors due to differences in aromatic residue accessibility. Accordingly, the F_{RGB} film operates as a total protein assay in which proteins contribute proportionally to their reactivity, and calibration with a representative whey protein enables reliable estimation of overall protein concentration rather than selective detection of a single species.

The applicability of the F_{RGB} method depends on the accessibility of soluble proteins to the diazotised surface. In whey and whey-derived concentrates, proteins remain dissolved and readily interact with the film, enabling direct measurement. In contrast, complex dairy matrices such as milk, yogurt or cheese may limit diffusion and would likely require specific pretreatment and independent validation. Therefore, the present study is intentionally restricted to whey and whey-derived protein concentrates, which correspond to the intended analytical application of the method.

To further validate the method, 13 whey samples with known protein concentrations (previously quantified by the Bradford assay) were analysed using both the A280 absorbance and RGB-based approaches (SM-Section S3, Figure S3). A linear correlation was obtained between both measurements ($R^2 = 0.97$; Figure 4b), demonstrating good agreement with the reference method and confirming accurate protein quantification across different samples.

To evaluate performance under realistic fraud scenarios, whey samples (5 mg mL^{-1} protein) were supplemented with representative non-protein nitrogen (NPN) sources, including free amino acids (glycine, leucine and glutamine), urea and an ammonium salt, while maintaining constant pH. Free amino acids were added simultaneously (2 mg mL^{-1} each; total 6 mg mL^{-1}), exceeding the native protein concentration ($\sim 120\%$), whereas urea and ammonium salt were individually added at concentrations equivalent to the protein content (5 mg mL^{-1}). Despite these severe adulteration conditions, the F_{RGB} response did not

change (Figure 4c), demonstrating that the signal depends on protein-bound aromatic residues rather than total nitrogen. In contrast, nitrogen-based methods may overestimate protein content in the presence of NPN compounds.⁷ Therefore, the F_{RGB} method can serve as a rapid preliminary authenticity screening prior to confirmatory laboratory analysis.

Unlike nitrogen-based methods such as Kjeldahl, which quantify total nitrogen regardless of its chemical form, the F_{RGB} response remained unchanged in the presence of non-protein nitrogen compounds. This confirms that the sensing mechanism is selective for protein-bound aromatic residues, enabling discrimination between true protein content and nitrogen-based adulteration.

3.5. Linearity calibration with commercial WPC using the F_{RGB} method

To evaluate applicability under real-use conditions, the F_{RGB} sensor was tested with two commercial whey protein concentrate (WPC) formulations (Evowhey Protein 2.0, biscuit and coffee-with-milk flavours; HSN). Based on the labelled protein contents (78 and 76 g/100 g, respectively), aqueous solutions covering 0–120 mg mL⁻¹ were prepared and analysed using the F_{RGB} procedure. RGB parameters obtained from film images were plotted against protein concentration to generate calibration curves for both products. Because commercial WPC supplements contain non-protein additives (sweeteners, flavourings and stabilisers), calibration was performed using the real formulation rather than purified protein solutions to ensure quantitative accuracy under practical conditions.

Figure 5 shows the response of the coffee-with-milk WPC sample. As previously observed for β -lactoglobulin and whey, the B parameter was linear only at low concentrations (0– $\approx$ 16 mg mL⁻¹) and reached a plateau at higher concentrations (Figure 5a), whereas the G parameter remained linear across the full evaluated range (0–120 mg mL⁻¹) (Figure 5b).

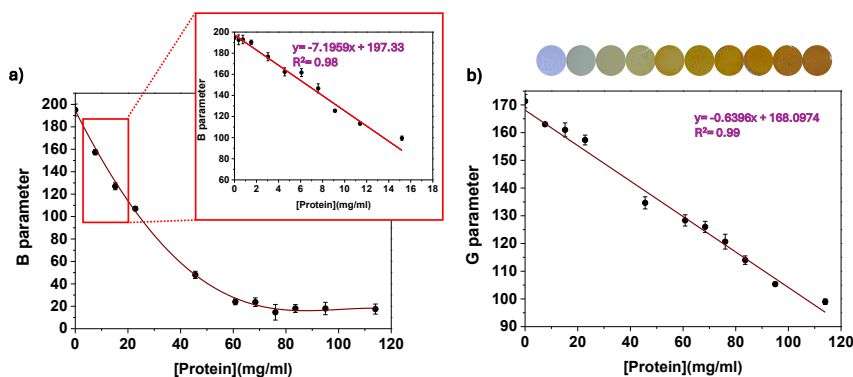


Figure 5. a) Calibration curve of Evowhey Protein 2.0 HSN (coffee with milk flavour) using the F_{RGB} method, plotting the B parameter from image analysis. b) Calibration line of Evowhey Protein 2.0 HSN (coffee with milk flavour) using the F_{RGB} method, plotting the G parameter. Real film images at each concentration of the commercial formula are also presented.

To further validate the method, an independent set of 14 WPC samples was analysed using both A280 spectrophotometry and the F_{RGB} method. Protein concentrations determined by both approaches showed good agreement, yielding a linear correlation ($R^2 = 0.97$; Figure 6c; SM-Section S3, Figure S4). These results confirm that the polymeric film provides reliable quantitative estimation of protein concentration in commercial formulations.

Analytical performance was further assessed in terms of precision, accuracy and reproducibility. Repeatability evaluated using three independent F_{RGB} discs ($n = 3$) yielded relative standard deviation (RSD) values below 5% for the B parameter. Accuracy was determined by spike–recovery experiments performed in a commercial WPC matrix, providing a recovery of $94 \pm 1.46\%$. Films synthesised on different days showed consistent calibration slopes and measured concentrations, indicating low inter-batch variability. Additional data are provided in SM-Section S4.

The biscuit-flavoured WPC exhibited comparable calibration behaviour (Figure 6), with early saturation of the B parameter and sustained linearity of the G parameter. The similarity of calibration profiles for both formulations indicates that flavouring additives do not interfere with the sensing response.

The F_{RGB} sensing mechanism is governed by a heterogeneous covalent azo-coupling reaction rather than by adsorption equilibria. Proteins diffuse through the aqueous phase to the polymer interface and react irreversibly with diazonium groups, whereas low-molecular-weight additives present in commercial WPC (sweeteners, flavourings or surfactants) lack activated aromatic residues and therefore cannot participate in the reaction. Consequently, these components do not chemically compete with proteins for sensing sites, explaining the comparable calibration behaviour observed across formulations.

As the azo-coupling reaction progresses, the film colour evolves from bluish to red tones. The absorption band of the azo chromophore first develops in the blue region of the visible spectrum (~ 450 nm), producing a rapid decrease and early saturation of the B channel. At higher protein concentrations the absorption extends toward longer wavelengths (~ 500 – 550 nm), while the G channel remains responsive. Consequently, the analytical response shifts from the high-sensitivity B channel at low concentrations to the G channel at higher concentrations, effectively extending the dynamic range of the method. Absorbance spectra of the reacted films (SM-Section S1, Figure S1a) confirm this behaviour and demonstrate that the B-channel plateau originates from optical saturation rather than depletion of reactive diazonium sites.

The F_{RGB} material therefore exhibits a dual-range analytical behaviour in which two colour channels provide linear responses in complementary concentration domains. The B channel enables sensitive quantification of dilute protein solutions, whereas the G channel allows direct measurement of concentrated WPC without dilution, expanding the working range of the method for on-site quality control.

Conventional colorimetric assays rely on a single analytical signal and therefore require dilution when analysing concentrated samples due to photometric saturation. In contrast, the F_{RGB} approach exploits two optical channels derived from the same chromogenic reaction. This strategy converts a saturable response into a multi-range quantitative measurement and enables

direct analysis of both dilute whey streams and highly concentrated WPC solutions.

The method can also be positioned relative to other portable protein detection approaches. Simple colorimetric strips are rapid but typically semi-quantitative, whereas portable spectrometers and electrochemical sensors offer higher sensitivity at the cost of dedicated instrumentation and operational complexity. The F_{RGB} method provides quantitative analysis using only a polymeric film and a smartphone image, with minimal sample preparation and very low cost per test. Although its detection limit ($\sim 0.284 \text{ mg mL}^{-1}$) is higher than laboratory reference techniques, it matches the concentration range encountered in whey and WPC and is therefore intended as a rapid screening and process-control tool rather than a replacement for laboratory methods.

Finally, the operational characteristics of the material were considered. The precursor films are stable during storage at room temperature when protected from light, and diazotisation is performed immediately before use to ensure reactivity of the diazonium groups. Because the sensing reaction is irreversible, the films are single-use; however, the material cost is low ($\sim 0.05\text{--}0.10 \text{ €}$ per test) and no specialised instrumentation is required beyond image acquisition, supporting routine on-site screening applications.

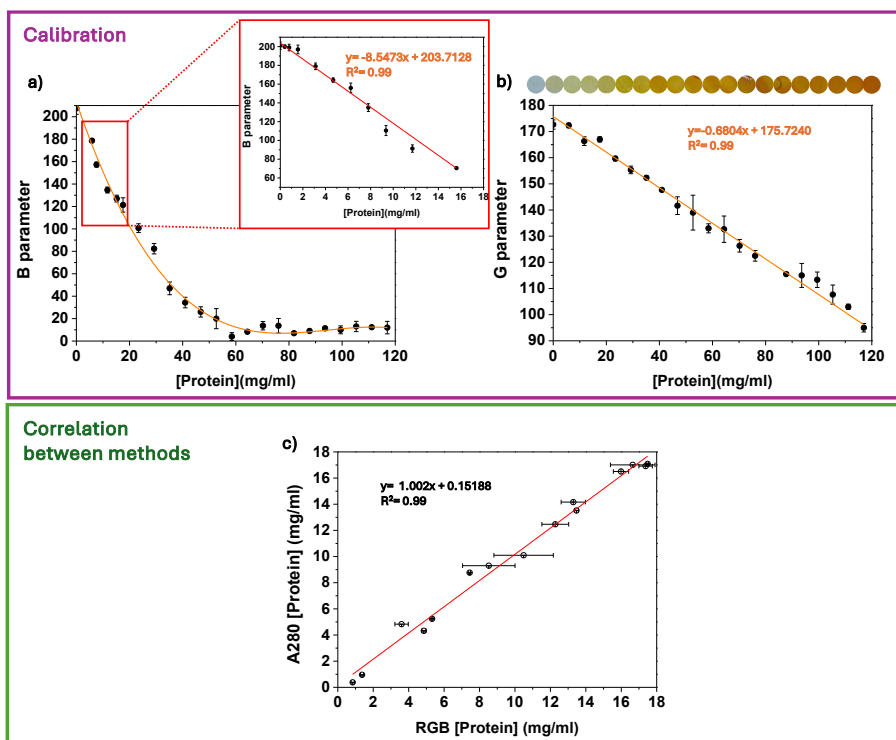


Figure 6. a) Calibration curve of Evowhey Protein 2.0 HSN (biscuit flavour) using the F_{RGB} method, plotting the B parameter from image analysis. b) Calibration line of Evowhey Protein 2.0 HSN (biscuit flavour) using the F_{RGB} method, plotting the G parameter. Real film images at each concentration of the commercial formula are also shown. c) Protein concentration (mg mL^{-1}) determined by the F_{RGB} and A280 methods in Evowhey Protein 2.0 HSN (biscuit flavour).

4. Conclusions

A polymeric film sensor based on diazonium–azo coupling (F_{RGB}) was developed for rapid protein quantification in whey and whey protein concentrates. The method operates as a solid-phase colorimetric assay read by smartphone imaging and is intended as a screening and process-control tool rather than a replacement for official laboratory methods.

The sensor showed consistent performance in real whey and commercial WPC samples and was not affected by lactose or non-protein nitrogen

compounds, enabling detection of adulteration scenarios that may compromise nitrogen-based assays.

A key feature of the system is its dual-range analytical behaviour: the blue channel provides high sensitivity at low protein concentrations, while the green channel remains linear at high concentrations. This complementary response allows direct measurement of both dilute whey and highly concentrated WPC without dilution using a single sensor and a single image acquisition.

Consequently, the F_{RGB} method enables rapid, low-cost and equipment-free estimation of protein concentration directly at the point of use. The approach is particularly suited to reception control, formulation adjustment and authenticity screening in whey-derived protein products, simplifying routine analysis under real production conditions. Because the sensing mechanism relies on covalent reaction with aromatic residues common to many proteins, the methodology could potentially be extended to other protein-containing matrices through matrix-specific calibration and optimisation of film composition and accessibility.

Data Availability

Open Data is available at <https://riubu.ubu.es/handle/10259/5684> under the name “UBU-Polymers Research Group 21032024”.

Competing interests

The authors declare no conflicts of interest.

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Supplementary Material

Additional information including physicochemical characterization, structural accessibility analysis and experimental comparison of whey proteins, calibration curves (A280 and B-parameter vs protein concentration), and analytical validation of the F_{RGB} method.

The Supplementary Material is available free of charge at:

<https://ars.els-cdn.com/content/image/1-s2.0-S0308814626011623-mmc2.pdf>

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*Smart polymers and smartphones for betalain measurement in cooked
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Smart polymers and smartphones for betalain measurement in cooked beetroots

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Abstract

Betalains in beetroots offer notable colouring properties and health benefits, including antioxidant, anti-inflammatory, hepatoprotective, and antitumorous activities. However, they degrade due to processing and storage conditions like temperature, pH, oxygen, and light-exposure. Traditional betalain determination methods are resource-intensive solid-liquid extractions. This study proposes a novel approach using a smart polymer to rapidly quantify betalains in processed beetroots. The polymer, containing *N,N*-dimethylaminoethyl methacrylate, selectively interacts with compounds like betalains. Characterization shows thermal stability over 250°C and suitable mechanical properties. The film changes to colour upon interaction with betalains, allowing quantification via smartphone. The sensory polymer's efficacy was validated across 27 beetroot samples, showing no significant differences compared to traditional methods. Combining the smart polymer with a colour analysis app, "Colorimetric Titration," provides a robust and efficient means of quantifying total betalains in beetroot puree, reducing the quantification time from 180 to 90 minutes, promising implications for routine food industry quality assessments.

Keywords: polyacrylic films; hydrogels; colourimetry; pigments; red beet;

1. Introduction

Betalains are water-soluble, nitrogen-containing pigments,¹ synthesized from the amino acid tyrosine and classified into two structural groups: betacyanins and betaxanthins (red-purple and yellow-orange, respectively).

The stability of betalains is affected by factors such as temperature, pH value, oxygen, and light.² Although pH changes cause alteration in their charges, betalains are relatively stable over the broad pH range from 3 to 7.³ Temperature is the most important factor in betalain stability during processing and storage² and the effects of high temperatures are increased with light exposure.⁴

However, the light-induced degradation of betalains can be minimized in the absence of oxygen.³ Interestingly, betaxanthins show greater stability than betacyanins under the mentioned conditions.⁴ In this sense, previous reports have suggested that the ideal conditions to preserve betacyanins are low temperatures at pH 5 in the dark.⁵ Betalains are restricted to the suborder Chenopodinae and can be found in roots, fruits and flowers among the Caryophyllales.¹ These pigments appear, therefore, in beetroots (*Beta vulgaris*), prickly pear fruits (*Opuntia* spp.), the red pitahaya or red dragon fruit (*Hylocereus polyrhizus*) and amaranth (*Amaranthus* sp.).⁶ Red beetroot (*Beta vulgaris*) has been reported to have more betalain content than other betalain-containing plants⁷ and it is the major commercially exploited betalain crop.⁸ It is widely grown in Europe, America, and Asia⁹, and although it is not consumed as much as other vegetables, beetroots have a significant market.¹⁰ Recently, the demand for healthy food has also increased consumer interest in red beetroot.¹¹ A large proportion of commercial production is processed into boiled and sterilized beetroots or canned into pickles.⁹

Betalains are responsible for the colour of these roots. At the point of purchase, the consumer uses appearance factors as quality indicators,¹² and beetroot colour is the most important quality indicator for consumer preference.^{9,13} Beetroot colour changes are directly related to betalain

degradation.¹⁴ Betalains can suffer degradation during storage or processing, leading to discolouration in beetroot.^{2,15} Betalains have also been described as bioactive compounds. In this sense, betanin acts as a free radical scavenger and an inducer of endogenous cellular enzymatic antioxidant defence mechanisms.¹³ Aside from their antioxidant activity, betalains have been described as having immunosuppressive, anti-inflammatory, hepatoprotective, and antitumorous biological activity.¹¹

Therefore, betalains have colouring properties and potential positive effects on human health. Given the dual role of betalains, and their instability upon temperature and storage, the determination of these bioactive pigments could be used as a sensory and functional quality marker for beetroot products. This is why the quantification of these compounds using simple and direct methods could have applications in the industry, both in quality control and in the potential prediction of the time it will take for the color to change, leading to consumer rejection.

Conventional methods for betalain determination are based on solid-liquid extractions,^{16–18} which are often unsustainable, due to the amount of organic solvents used, time-consuming, expensive, and require infrastructure and highly specialized equipment. These drawbacks can make implementing this analysis into routine food industry quality assessments challenging.

In this sense, smart polymers, particularly as films, are emerging as a robust solution well-received within the agri-food domain. Recent literature¹⁹ shows smart polymers can be categorized into two primary groups: reactive polymers and sensory polymers. The former instigates specific actions upon encountering a target (e.g., controlled drug release, alterations in shape), while the latter prompts an alert upon target detection, often manifested through changes in colour or fluorescence.

This study proposes a sensory polymer that selectively interacts with targets exhibiting formal negative charges in their structure, such as betalains. In the case of betalains, the negative charges are provided by the carboxylic acid

groups present in their chemical structure (Figure 1), which have a pKa of approximately 2, and therefore, are deprotonated at the typical pH of beetroots (from 5.5 to 6.6). These light-sensitive coloured compounds become trapped within the structure of the polymeric material, resulting in two relevant outcomes. Firstly, they induce a colour change in the material, enabling quantification through colour analysis. Secondly, the polymeric chains create a protective environment that prevents the degradation of such compounds, as demonstrated in prior studies,^{20,21} at least for the duration of the analysis. This facilitates their potential utilization in industrial settings, obviating the need for a dark environment. The use of a smartphone as complementary equipment has been significantly enhanced in recent years, which boasts high-quality cameras and processors that complement sensory polymers perfectly, democratizing chemical analysis and making it more efficient and cost-effective.²²

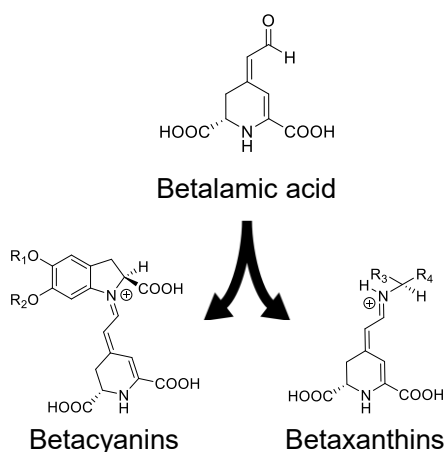


Figure 1. Chemical structure of the two types of betalains, betacyanins and betaxanthins, as well as the chemical structure from which they derive, betalamic acid.

2. Experimental

2.1. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: methylmethacrylate (**MMA**) (Aldrich, 99%), *N,N*-dimethylaminoethyl metacrylate (**NNDA**) (Aldrich, 98%), 2-hydroxyethyl acrylate (**2HEA**), (Aldrich, 96%), ethylene glycol dimethacrylate (**E**) (Aldrich, 98%), hydrochloric acid (VWR, 37%), acetone (VWR, 99%), KH_2PO_4 (Scharlau, 99%), Na_2HPO_4 (Merck, 99%), ethanol (VWR/BDH Chemicals, 96%). Azo-bis-isobutyronitrile (AIBN, Aldrich, 99%) was recrystallized twice from methanol.

Beetroot samples were both provided by HUERCASA 5ª GAMA S.A. company and purchased in different local markets.

2.2. Instrumentation and general methods

The thermal characterization of the smart polymer was conducted utilizing various analytical techniques. For the adhesives, thermogravimetric analysis (TGA) was performed employing a Q50 TGA analyzer (TA Instruments, New Castle, USA). Samples weighing 3–7 mg were subjected to a nitrogen atmosphere with a heating rate of $10^\circ\text{C}\cdot\text{min}^{-1}$.

Tensile properties analysis, with $5 \times 9.44 \times 0.103$ mm samples tested at 5 mm min^{-1} (EZ Test Compact Table-Top Universal Tester, Shimadzu Kyoto, Japan).

Infrared spectra (FTIR) were recorded using an infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) equipped with an ATR-PRO410-S single reflection accessory.

The water swelling percentage (WSP), was performed in triplicate, and calculated using the following equation.

$$WSP = \left[\frac{g_{\text{swelled material}} - g_{\text{dried material}}}{g_{\text{dried material}}} \right] \times 100 \quad (\text{eq. 1})$$

2.3. Design and synthesis of the film-like smart polymer

For a correct and efficient polymeric material design, it is necessary to consider its final use and the type of users handling it. We aim for this development to be usable by non-specialized personnel and under analysis conditions in industrial environments, or in other words, in environments that are not typical laboratories. That is why we have chosen the film format for our materials, as it can be manipulated by hand without filtration or extraction processes. Additionally, the selected formulation generates a material with gel-like behaviour, meaning it swells in aqueous media, allowing the target species (betalains) to enter the polymeric structure and interact with the material's receptor centres.

The film-like smart polymer (FiLiPo) was prepared by thermally initiated bulk radical polymerization.^{23,24} In this experimental procedure, three commercially available monomers, NNDA, 2HEA, and MMA were copolymerized in a specific molar ratio 45/45/10, respectively. The function of NNDA is twofold, as it serves both as a monomer that imparts hydrophilicity to the material and as the active monomer that, after a process of protonation of the tertiary amine group, allows the generation of positive charges in the polymer (polycation). Considering that betalains are species with formal positive and negative charges, our detection strategy is based on electrostatic interactions with the polymer through these charges. The 2HEA serves a structural function in the material, providing hydrophilicity and some flexibility. On the other hand, MMA contributes to hydrophobicity and rigidity.

The combination of these monomers generates a material with high hydrophilicity, resulting in a high WSP and, therefore, poor handling. To control swelling, we have incorporated ethyleneglycol dimethacrylate as crosslinking agent. In fact, we have added 15 mol% of crosslinking agent, which is a high amount compared to other materials we have published.^{24–27} The proper balance of monomers and crosslinker enables control over the intricate equilibrium between handling, WSP, and the speed of interaction between the target and smart polymer.

Finally, a 1 w% of AIBN (thermal radical initiator), was added to the mixture of monomers and crosslinker, and the resulting solution was injected into a mould (100 μm thickness). The oxygen-free polymerization was carried out at 60 °C overnight. The solid film was demoulded, and dipped into different solutions to both remove nonreacting monomers, and aconditioning for the measurement of betalains. Specifically, it was first immersed in distilled water for 15 minutes, and then progressively replaced with acetone until reaching 100% acetone. Conversely, acetone was gradually replaced with distilled water until returning to a 100% aqueous medium. This progressive solvent exchange is necessary to avoid abrupt changes in the swelling of the material that could cause film ruptures. Finally, the film was immersed in an aqueous solution of HCl (4%) to protonate the tertiary amine groups contained in the NNDA monomer, thereby generating positive charges in the polymeric chain and enabling electrostatic interaction with betalains. Finally, the material was cut into discs with an 8 mm diameter.

2.4. Preparation of calibration samples (CS)

As previously described in the literature,¹⁴ the betalain content of beetroots decreases as the temperature increases. In this study, we have utilized this phenomenon to construct a calibration curve that allows the determination of the concentration of betalains using the FiLiPo method. In order to achieve a wide range of betalain concentrations, *Beta vulgaris* cv. Monty was the cultivar selected due to its high betalain content.^{28–30}

Eight kg of fresh beetroots were washed, dried, peeled and chopped into similar-sized slices. The sliced beetroot was vacuum-packed in plastic bags (250 g per bag). Then, samples were boiled, and two bags were taken out at different boiling times (from 0.5 to 7.5 h). Beetroot slices were immediately grinded in a blender to obtain a puree.

2.5. Extraction of betalains from beetroots

A betalain extraction was performed as described elsewhere.¹⁴ Briefly, 0.5 g of each puree sample were diluted with 5 ml ethanol-water solution (50:50 v/v) and immediately shaken (10 min, RT) and centrifuged (10 min, 4°C, 5500 rpm). Then, the supernatant was collected. Two consecutive extraction cycles were performed by repeating the same procedure. Extracts were stored at -30°C until analysis. The extraction was performed in triplicate. Samples were carefully handled along the procedure to minimize light exposure.

2.6. Determination of betalain content. Traditional Method.

Betalain content was determined by spectrophotometry (UV/Vis) following the method described elsewhere.³¹ The extract was diluted with a phosphate buffer (pH 6.5) until the absorbance at 538 nm was between 0.4–0.5. The absorbance of the samples was measured at 538 nm and 476 nm to quantify betacyanins and betaxanthins, respectively. Absorbance at 600 nm was recorded to correct for small amounts of impurities. Betacyanin and betaxanthin concentrations were expressed in mg/kg. Total betalain content was calculated as the sum of betacyanins and betaxanthins.

2.7. Determination of betalain content in beetroots using FiLiPo and the app “Colorimetric Titration”

Two grams of the beetroots' purees were weighed into 5 mL vials, and an 8 mm diameter FiLiPo disc was introduced for 1 hour at room temperature ($20 \pm 1^\circ\text{C}$, 3 replicates). After this time, the discs were removed from the beetroots' purees, the surface was cleaned with a little distilled water, and they were photographed with an iPhone 11 Pro inside a backlit lightbox. The use of a backlight box helps maintain consistent lighting conditions, thus positively contributing to the reproducibility of the method. The colour of the FiLiPo discs was analyzed using the "Colorimetric Titration" app,³² and the colour parameters were extracted from two different digital colour spaces, the RGB colour space (the most common in electronic devices) and the HSV colour space. Additionally, two different

combinations of RGB parameters were analyzed. In total, 8 different digital colour variables were examined.²²

After analyzing the 12 calibration samples (Section 2.4), one specific colour parameter was chosen for the calculations, namely the one that exhibited the best correlation with the betalain content calculated using the traditional method. Subsequently, the 27 beetroot puree samples were analyzed using the FiLiPo method (Figure 2), and the betalain concentration was calculated using the obtained colour parameters from digital photographs and the calibration curve equation.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism v8 software. To evaluate the statistical significance of the correlation between total betalains derived from the reference method and the different color parameters obtained with the FiLiPo method, the normality of the data of the calibration samples was analyzed and a Pearson product-moment correlation test was performed. A correlation coefficient (r) value close to 1 or -1 and a $p < 0.05$ described a positive or negative correlation. To compare the betalain content of the samples obtained by the traditional method and FiLiPo-based method, the normality of the data of the test samples was analyzed and a non-parametric Mann-Whitney test was used ($p < 0.05$).

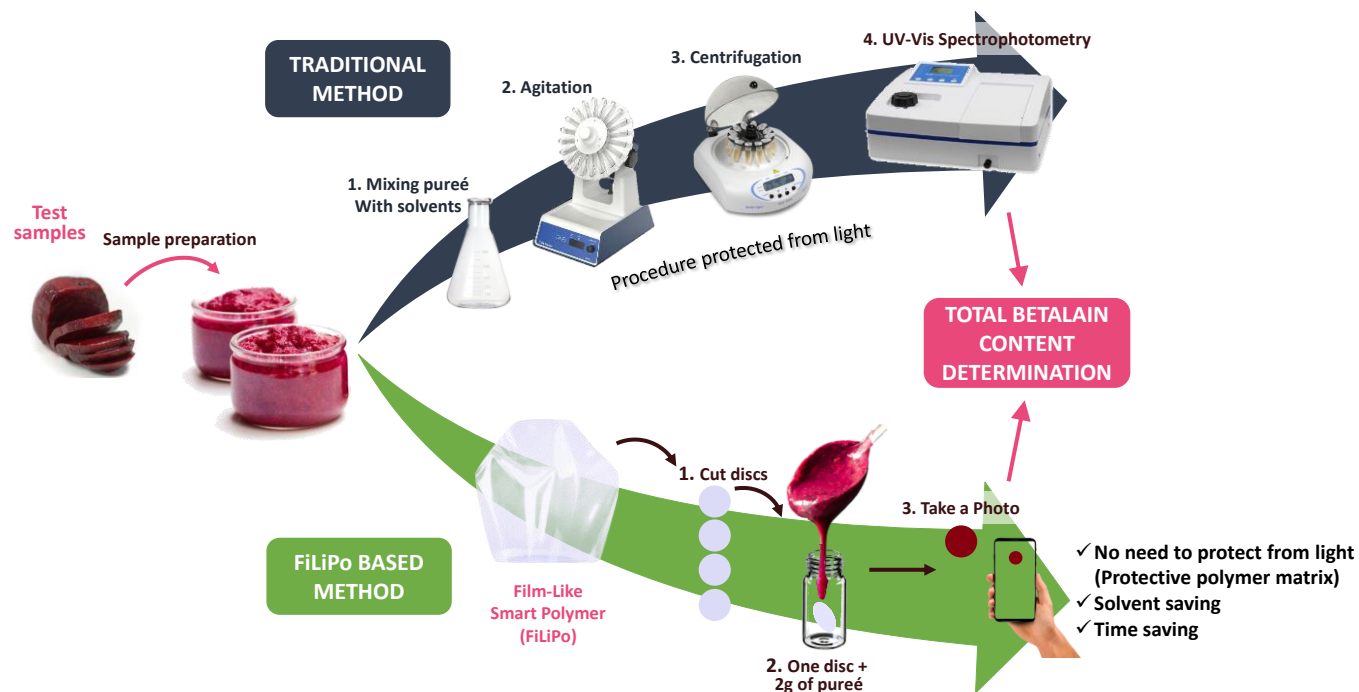


Figure 2. Table of contents

3. Results and discussion

3.1. Characterization of the film-like smart polymer

Characterizing the handling of such materials is challenging, and often, it is necessary to manipulate and test them under real conditions to determine if they can withstand the process without breaking. There is not a specific technique that indicates this handling, but rather a combination of various techniques, such as the evaluation of their thermal and mechanical properties. Figure 3 illustrates the proposed material's chemical structure and part of its thermal characterization and infrared analysis. Regarding the thermal behaviour of FiLiPo (Figure 3b), we can observe that it has a T_5 and a T_{10} of 254 °C and 272 °C, respectively (temperatures at which 5% and 10% of the material weight is lost due to thermal degradation). For this application, it is relevant that we do not have significant weight loss below 100°C, as the material could potentially come into contact with cooking water, or freshly cooked beetroots, which may reach around that temperature.

The FT-IR analysis confirmed the polymer's structure and revealed characteristic bands of the three different monomers (Figure 3c). For instance, the broadband observed at 3,372 cm^{-1} is indicative of the vibration VO-H in **2HEA**, the band at around 1,233 cm^{-1} corresponds to the VC-N vibration in **NNDA**, and the bands observed at 2,950 cm^{-1} and 1,718 cm^{-1} are attributed to the C-H vibration of the sp^3 carbons and the VC=O vibration, respectively, present in all the monomers.

The WSP was found to be 55%, a common value in gel-like films used in the detection of targets in food matrices.^{24,33} Typically, with increased swelling, the response speed of the material tends to rise; however, the polymer matrix absorbs much more water, impacting the material's mechanical properties. Thus, once again, there is an effort to balance swelling and mechanical properties.

The Young's modulus of the material was measured immediately after being demoulded in the FiLiPo preparation process, yielding a value of 291.5 ± 1.4 MPa. This value is not unusual for this type of material.^{21,34} However, it could be improved in future applications, especially by increasing the molar proportion of MMA, albeit sacrificing some of the hydrophilicity provided by 2HEA.

Given that this material is proposed as an option for use in the food industry, it is important to highlight that it has been designed with biocompatible monomers such as [2-(methacryloyloxy)ethyl]trimethylammonium chloride,³⁵ 2-hydroxyethyl acrylate (used in the manufacture of contact lenses),³⁶ and methyl methacrylate (used in the production of dental prosthetic).³⁷ Moreover, the risk of compound migration into the food matrix is negligible, as demonstrated in previous studies.³⁸ These studies have shown that the migration of substances from similar materials into food matrices with which they come into contact is minimal, complying with European Union regulations on food contact (Regulation (EU) No 10/2011 and amendments).

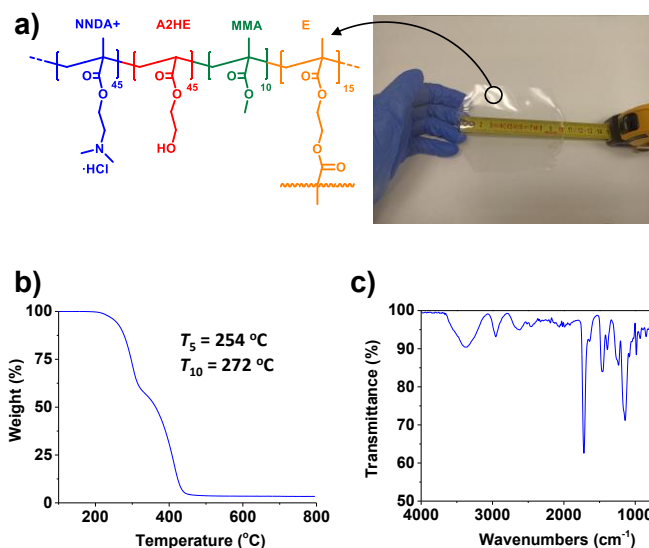


Figure 3. Structure and properties of the FiLiPo material. a) Chemical structure of the Film-Like Smart Polymer (FiLiPo); b) thermogravimetric curves of FiLiPo at 10 °C/min under a nitrogen atmosphere, displaying T_5 and T_{10} temperatures; c) FT-IR spectrum of FiLiPo.

3.2. Results of betalain content and RGB parameters of the calibration samples.

Table 1 displays the total betalain content results of the 12 calibration samples, calculated using the traditional method described in Section 2.6. Furthermore, Table 1 presents the different colour parameters obtained through the analysis of the FiLiPo discs using the "Colorimetric Titration" smartphone application. These parameters are extracted from the analysis of the photographs taken of the FiLiPo discs shown on the right side of the table. At the bottom, a brief correlation study is displayed between each of the 8 analyzed colour variables and the total betalain concentration data obtained by the traditional method.

The results show that the best correlation is with the parameter "R" of the RGB colour space, thus, we constructed our calibration curve by plotting this parameter against the concentration of total betalains (Figure 4).

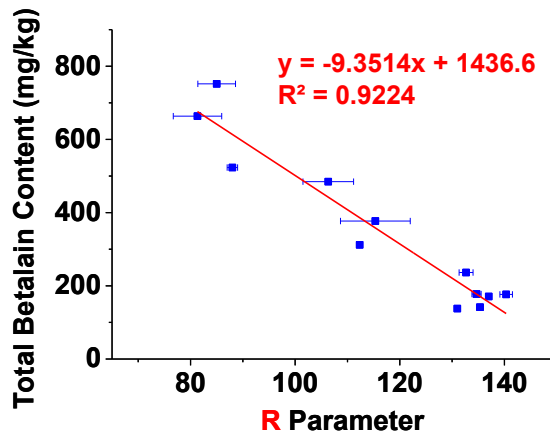


Figure 4. Calibration curve. The betalain content was calculated according to the method described in Section 2.6. The "R" parameter of digital colour was extracted from photos of 8 mm diameter FiLiPo discs after being immersed for 1 hour in beetroots purees at room temperature. The data show the standard error of 3 replicates.

Table 1. Results of betalain content in the calibration samples obtained by the traditional method (in blue), and results of the 8 digital colour variables extracted from the photographs taken of the FiLiPo discs (green). Table shows cropped images of the FiLiPo discs (8 mm in diameter) after being immersed for 1 hour in 2 grams of calibration samples' purees at $20 \pm 1^\circ\text{C}$. The bottom part of the table shows the Pearson correlation coefficient (r) and the coefficient R2 between both methods taking into account the different digital colour parameters. Data are means of 3 replicates $\pm$ standard error.

Calibration Sample (CS)	Trad. Method	FiLiPo Method										
	Total Betalain (mg/kg)	R	G	B	ΔRGB	RGB	H	S	V	Cropped images of the FiLiPo discs (Rep. 1)	Cropped images of the FiLiPo discs (Rep. 2)	Cropped images of the FiLiPo discs (Rep. 3)
CS#1	751.95 $\pm$ 1.70	85.00 $\pm$ 3.61	17.00 $\pm$ 3.51	51.33 $\pm$ 3.48	356.46 $\pm$ 4.34	-0.777 $\pm$ 0.11	0.915 $\pm$ 0.00	0.798 $\pm$ 0.04	0.333 $\pm$ 0.01			
CS#2	663.59 $\pm$ 4.58	81.33 $\pm$ 4.63	2.33 $\pm$ 0.88	34.00 $\pm$ 5.03	377.99 $\pm$ 5.51	-1.250 $\pm$ 0.17	0.934 $\pm$ 0.01	0.972 $\pm$ 0.01	0.319 $\pm$ 0.02			
CS#3	523.17 $\pm$ 1.99	88.00 $\pm$ 1.00	2.33 $\pm$ 1.86	40.33 $\pm$ 1.45	371.24 $\pm$ 1.65	-2.008 $\pm$ 0.01	0.925 $\pm$ 0.00	0.973 $\pm$ 0.02	0.345 $\pm$ 0.00			
CS#4	484.69 $\pm$ 2.87	106.33 $\pm$ 4.84	3.00 $\pm$ 0.58	66.00 $\pm$ 9.50	348.50 $\pm$ 7.14	-0.274 $\pm$ 0.23	0.899 $\pm$ 0.01	0.972 $\pm$ 0.01	0.417 $\pm$ 0.02			
CS#5	377.06 $\pm$ 0.61	115.33 $\pm$ 6.67	18.67 $\pm$ 12.68	103.33 $\pm$ 3.48	314.25 $\pm$ 4.86	0.065 $\pm$ 0.17	0.853 $\pm$ 0.00	0.823 $\pm$ 0.13	0.452 $\pm$ 0.03			
CS#6	312.04 $\pm$ 5.89	112.33 $\pm$ 0.33	24.33 $\pm$ 0.88	100.00 $\pm$ 1.00	312.39 $\pm$ 1.28	-0.040 $\pm$ 0.04	0.857 $\pm$ 0.00	0.783 $\pm$ 0.01	0.441 $\pm$ 0.00			
CS#7	236.42 $\pm$ 3.36	132.67 $\pm$ 1.33	4.33 $\pm$ 1.76	118.33 $\pm$ 3.28	310.62 $\pm$ 3.38	0.989 $\pm$ 0.09	0.852 $\pm$ 0.00	0.968 $\pm$ 0.01	0.520 $\pm$ 0.01			

Calibration Sample (CS)	Trad. Method	FiLiPo Method										
	Total Betalain (mg/kg)	R	G	B	Δ RGB	RGB	H	S	V	Cropped images of the FiLiPo discs (Rep. 1)	Cropped images of the FiLiPo discs (Rep. 2)	Cropped images of the FiLiPo discs (Rep. 3)
CS#8	177.53 ± 1.27	134.67 ± 0.88	32.33 ± 1.20	155.00 ± 1.15	272.15 ± 1.30	0.632 ± 0.03	0.806 ± 0.00	0.791 ± 0.01	0.608 ± 0.00			
CS#9	176.61 ± 1.17	140.33 ± 1.20	16.00 ± 3.79	138.33 ± 4.26	289.64 ± 5.23	0.004 ± 0.13	0.836 ± 0.00	0.887 ± 0.03	0.554 ± 0.01			
CS#10	170.93 ± 1.43	137.00 ± 0.00	15.67 ± 1.20	136.67 ± 1.20	291.91 ± 1.47	0.049 ± 0.04	0.833 ± 0.00	0.886 ± 0.01	0.540 ± 0.00			
CS#11	141.65 ± 1.76	135.33 ± 0.67	34.00 ± 0.58	143.00 ± 1.00	275.15 ± 0.38	0.542 ± 0.02	0.822 ± 0.00	0.762 ± 0.01	0.561 ± 0.00			
CS#12	137.86 ± 0.69	131.00 ± 0.58	64.67 ± 3.33	162.33 ± 5.70	245.42 ± 4.79	0.907 ± 0.14	0.781 ± 0.01	0.602 ± 0.01	0.637 ± 0.02			
CORRELATION OF TRADITIONAL METHOD WITH THE DIGITAL COLOUR PARAMETERS												
		R	G	B	Δ RGB	RGB	H	S	V			
Pearson Coefficient (r)		-0.960	-0.557	-0.943	0.912	-0.779	0.917	0.418	-0.942			
R ²		0.922	0.310	0.890	0.832	0.607	0.841	0.175	0.888			

3.3. Results of betalain content of the test samples by both methods

A total of 27 beetroot purees were measured using the traditional and FiLiPo-based methods. For the latter, the parameters "R" obtained from the photographs allowed the calculation of betalain concentration through the calibration curve equation, as shown in

Table 2. Results of betalain content in the test samples obtained by the traditional method (in blue), and FiLiPo-based method (green). Data are means of 3 replicates $\pm$ standard error. Statistical analysis: Mann Whitney test ($p < 0.05$); "ns" means no significant differences.

Beetroot purees	Traditional Method (mg/kg)	FiLiPo-based Method (mg/kg)	Statistical analysis	Cropped images of the FiLiPo discs		
				Replicate 1	Replicate 2	Replicate 3
BRP#1	649.33 $\pm$ 10.11	604.33 $\pm$ 10.80	ns			
BRP#2	706.75 $\pm$ 6.87	729.01 $\pm$ 8.25	ns			
BRP#3	250.83 $\pm$ 4.69	220.92 $\pm$ 28.57	ns			
BRP#4	445.06 $\pm$ 0.51	336.25 $\pm$ 3.12	ns			
BRP#5	586.38 $\pm$ 2.77	566.92 $\pm$ 42.17	ns			
BRP#6	375.00 $\pm$ 1.81	270.79 $\pm$ 27.71	ns			
BRP#7	404.91 $\pm$ 3.28	389.24 $\pm$ 10.80	ns			
BRP#8	571.95 $\pm$ 3.62	492.11 $\pm$ 65.46	ns			
BRP#9	471.33 $\pm$ 3.03	367.42 $\pm$ 3.12	ns			
BRP#10	405.81 $\pm$ 1.01	345.60 $\pm$ 11.24	ns			
BRP#11	402.79 $\pm$ 3.59	420.41 $\pm$ 16.49	ns			

Beetroot purees	Traditional Method (mg/kg)	FiLiPo-based Method (mg/kg)	Statistical analysis	Cropped images of the FiLiPo discs		
				Replicate 1	Replicate 2	Replicate 3
BRP#12	180.45 ±0.31	183.51 ±5.40	ns			
BRP#13	368.56 ±1.85	389.24 ±16.20	ns			
BRP#14	370.88 ±2.13	370.54 ±5.40	ns			
BRP#15	175.59 ±0.82	180.40 ± 8.25	ns			
BRP#16	846.82 ±4.82	834.99 ±17.36	ns			
BRP#17	286.33 ±1.17	305.08 ±14.28	ns			
BRP#18	453.72 ±2.95	389.24 ±21.60	ns			
BRP#19	776.50 ±2.83	800.70 ±23.53	ns			
BRP#20	405.38 ±2.57	308.20 ±40.52	ns			
BRP#21	492.70 ±1.57	551.33 ±18.96	ns			
BRP#22	448.38 ±1.94	492.11 ±30.06	ns			
BRP#23	223.26 ±0.39	192.86 ±5.40	ns			
BRP#24	474.88 ±1.66	501.46 ±53.99	ns			
BRP#25	727.80 ±1.75	753.95 ±28.05	ns			
BRP#26	453.65 ±1.72	414.18 ±25.51	ns			
BRP#27	387.56 ±2.44	414.18 ±13.59	ns			

The results indicate that there are no statistically significant differences between the total values of betalains obtained with both methods. The statistical test utilized did not find significant differences, although some slightly different data were observed, such as in the case of sample 4 or sample 6.

Upon plotting the betalain content data obtained by both methodologies, we observe a strong visual correlation between the two, as depicted in Figure 5.

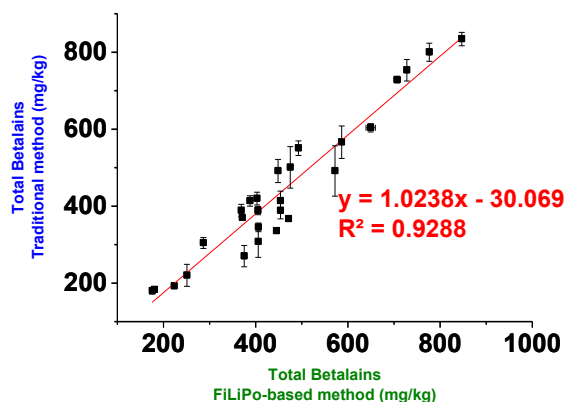


Figure 5. Total betalains calculated from traditional method and FiLiPo-based method. Data are means of 3 replicates $\pm$ standard error of the total betalains for each sample obtained with each method. The trendline illustrates how the results obtained with both methods correlate with each other, with an R^2 value of 0.93

This demonstrates that the utilization of the smart polymer FiLiPo, complemented by a colour analysis application such as "Colorimetric Titration," constitutes a robust method for quantifying total betalains in beetroot puree samples. This approach not only saves costs on equipment but also streamlines execution time significantly. The experimental duration per sample measurement is reduced from 180 minutes to 90 minutes, and the assay can be conducted by non-specialized personnel.

4. Conclusions

Our study presents a novel advancement in the quantification of betalains in processed beetroot, showcasing the efficacy of an innovative smart sensory

polymer film. This cutting-edge approach not only addresses the limitations of traditional methods but also introduces several significant advantages. Firstly, unlike conventional techniques that require working in dark conditions to prevent betalain degradation, our method eliminates this necessity by providing a protective environment within the polymer, thus simplifying the analytical process and enhancing convenience. Additionally, by avoiding the need for extensive solid-liquid extractions, our method significantly reduces the consumption of organic solvents, which contributes to sustainability efforts in analytical chemistry and reduces environmental impact. Moreover, the smart sensory polymer film exhibits a rapid and selective color change in response to betalains, enabling swift and precise quantification using ubiquitous devices such as smartphones. This feature eliminates the need for specialized instrumentation and trained personnel, making the process more accessible and cost-effective. The ease of use and reduced dependency on sophisticated equipment offer a practical solution for routine quality assessments in the food industry. Statistical analysis confirms the reliability and accuracy of our method, demonstrating no significant differences in betalain concentrations compared to traditional quantification approaches. This finding underscores the robustness of our methodology and validates its suitability for widespread adoption in quality control processes. In summary, the adoption of this smart polymer-based approach, complemented by color analysis applications, holds transformative potential for accelerating betalain quantification processes. This innovation facilitates more efficient, sustainable, and cost-effective quality control measures in the production of beetroot-derived products, offering a significant advancement in analytical techniques within the food industry.

Supplementary Material

Digital colour parameters of Calibration and Test samples.

Open Data

Open Data is available at <https://riubu.ubu.es/handle/10259/5684> under the name "UBU-Polymers Research Group 16042024".

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Supplementary Material

Digital colour parameters of Calibration and Test samples

The Supplementary Material is available free of charge at:

<https://ars.els-cdn.com/content/image/1-s2.0-S0308814624020089-mmc1.pdf>

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*Covalent immobilization of the DPPH molecule in reusable smart polymers
for the assessment of antioxidant capacity in beetroots*

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Covalent immobilization of the DPPH molecule in reusable smart polymers for the assessment of antioxidant capacity in beetroots

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Abstract

Antioxidants are used as markers of the functional potential of foods, making their accurate quantification essential for assessing bioactivity and possible health benefits. A smart polymer incorporating 2,2-diphenyl-1-picrylhydrazyl (DPPH) motifs (F_{DPPH}) was synthesized via bulk polymerization of N-vinylpyrrolidone, methyl methacrylate, and a diphenylamine-functionalized monomer, followed by solid-phase reactions to introduce the DPPH functionality. FTIR and thermal analysis confirmed successful synthesis. The F_{DPPH} sensor showed a colorimetric response consistent with DPPH in solution, demonstrating its reliability. Twenty-three beet samples were analyzed using both the conventional DPPH assay and the F_{DPPH} method, where polymer discs were immersed and colour changes recorded via smartphone. The hue (H) parameter in the HSV colour-space showed the highest correlation. Calibration using beet samples was more accurate than with Trolox. The optimal response time was 3 hours, with a LOD and LOQ of 0.063 and 0.191 mmol Trolox/L, respectively.

Keywords: polyacrylic films; sensory polymers; beetroot; food industry; colorimetric detection; smartphone-based analysis; antioxidant capacity; food quality control.

1. Introduction

Simple analytical methods, accessible to the general public and based on the combination of advanced materials and smartphones, have become an increasingly important topic in both scientific and social contexts. While many of these smartphone-based methods do not aim to match the sensitivity or selectivity of established analytical techniques, numerous approaches still fail to meet market and industry expectations.¹ In this study, we collaborate with the food industry to merge the reliability of well-established analytical methods with the latest smartphone-based chemical analysis trends. As a model application, we focus on the antioxidant capacity of beets, adapting the well-known 2,2-diphenyl-1-picrylhydrazyl (DPPH) method² for this purpose. Our choice of beetroot as the target is due to the high antioxidant capacity of this vegetable³ and influenced by the strong connection between our research group and the beet industry.⁴

The study of antioxidant capacity in food has a long and rich history spanning several decades. Initially, the focus was on identifying natural compounds capable of counteracting oxidative damage, a phenomenon linked to aging and numerous diseases. Over time, scientists developed various assays to quantify antioxidant activity, beginning with simple chemical tests and evolving into sophisticated analytical methods. The DPPH assay, introduced in the 1950s, has become one of the most widely used techniques due to its simplicity, speed, and reliability. Other assays such as the Oxygen Radical Absorbance Capacity (ORAC), Ferric Reducing Antioxidant Power (FRAP), and ABTS have further expanded the toolbox for antioxidant measurement, each with distinct mechanisms and advantages.^{5,6}

Despite its widespread use, the traditional DPPH assay faces several challenges when applied in real industrial food settings. These include the requirement for organic solvents, relatively long reaction times, and difficulties in adapting the method for on-site or rapid testing. Additionally, the assay's reliance on laboratory instruments limits its practical use for continuous quality control in food production environments. These limitations underscore the need for more accessible, faster, and user-friendly alternatives that maintain analytical reliability.

The integration of smartphones and the design of smart polymers overcome the barriers identified in traditional DPPH assays by eliminating the need for organic solvents and specialized laboratory equipment. Thanks to these advances, faster, simpler, and more portable detection is achieved, facilitating in situ testing and continuous quality control in real industrial environments, directly addressing the demands for accessibility and speed in the food industry.

Some recent innovations have sought to overcome this barrier. For instance, one approach utilizes a paper-based device shaped like a thermometer, which uses nanoceria to detect antioxidants. The colour change along the detection channel correlates with antioxidant capacity and can be measured using a simple integrated ruler, eliminating the need for sophisticated instruments.⁷ Another method employs immobilized ceria nanoparticles that change colour upon interacting with antioxidants, and these optical changes can be monitored using a smartphone camera, providing sensitive detection of common antioxidant compounds.⁸

In the food industry, another type of material being increasingly used consists of film-shaped sensory polymers that change colour, not only for the determination of antioxidant capacity but also for the quantification of various compounds of industrial interest,^{9–11} such as, iron,¹² copper,¹³ polyphenols,¹⁴ betalains,⁴ etc. These films are easy to use, and when combined with new generations of smartphones and image analysis software,¹⁵ they represent a precise and adaptable tool for detecting multiple targets. These features make

smart polymeric sensors ideal for transferring well-known laboratory assays, such as DPPH, into practical tools suitable for quality assurance in food production chains

In this study, we combine the robustness of the DPPH method—a well-established analytical technique for determining antioxidant capacity in foods—with the latest trends in accessible, smartphone-based methods that utilize sensor polymers. To achieve this, we chemically modified the DPPH molecule to covalently anchor it to a polymeric chain. This modification allowed the fabrication of a film-shaped material containing DPPH motifs, making the experimental procedure as simple as immersing the film in the sample, waiting, and capturing a photo. To date, no study has proposed a DPPH-based polymeric sensor that integrates covalently bound active motifs and is specifically designed for colorimetric detection via smartphone in a real-world food industry context. The covalent anchoring prevents the release or migration of free DPPH molecules under normal usage conditions, thereby minimizing any potential safety risks. Migration studies conducted on similar polymeric materials with covalently bound motifs have shown negligible migration and comply with relevant food contact regulations (Commission Regulation (EU) no. 10/2011, and amendments).¹⁰

2. Experimental

2.1. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: *N*-phenyl-*p*-phenylenediamine (TCI, >98%); methacrylic anhydride (Alfa Aesar, 94%); diethyl ether (VWR, 100%); hexane (distilled from VWR, mixture of isomers); *N*-vinylpyrrolidone (**VP**) (TCI, >99%); methylmethacrylate (**MMA**) (Aldrich, 99%), ethylene glycol dimethacrylate (**E**) (Aldrich, 98%), hydrochloric acid (VWR, 37%), sodium nitrite (Thermo Scientific, 98%); tin(II) chloride dihydrate (Alfa Aesar, 98%); chlorobenzene (TCI, >98%); sulfuric acid (VWR, 95%); fuming nitric acid (Merck, 100 %); toluene (VWR, 100%); benzoyl peroxide (Sigma Aldrich, with 25% of water); sodium sulfate (Sulquisa Minersa Group); sodium bicarbonate (VWR, 100%); dichloromethane (VWR, 100%); methanol (VWR, 100%); acetone (VWR, 99%), 1,1-diphenyl-2-picrylhydrazyl free radical (TCI, >97.0%). Azo-bis-isobutyronitrile (AIBN, Aldrich, 99%) was recrystallized twice from methanol.

Beetroot samples were both provided by HUERCASA 5ª GAMA S.A. company and purchased in different local markets.

2.2. Instrumentation and general methods

The thermal properties of the film-shaped polymers were assessed using a range of analytical methods. For the adhesives, Thermogravimetric Analysis (TGA) was conducted with a Q50 TGA analyzer (TA Instruments, New Castle, USA). Samples weighing between 3 and 7 mg were analyzed in a nitrogen atmosphere at a heating rate of 10°C/min. Differential Scanning Calorimetry (DSC) was performed using a Q200 DSC analyzer (TA Instruments, New Castle, USA), with a heating rate of 20°C/min under a nitrogen atmosphere, using samples weighing 10-15 mg.

Fourier Transform Infrared spectra (FTIR) were obtained with an FT/IR-4200 infrared spectrometer (FT/IR-4200, Jasco, Tokyo, Japan) equipped with an Attenuated Total Reflectance ATR-PRO410-S accessory.

The Water Swelling Percentage (WSP) was measured in triplicate and calculated using the Equation 1:

$$WSP = \left[\frac{g_{\text{swelled material}} - g_{\text{dried material}}}{g_{\text{dried material}}} \right] \times 100 \quad (\text{eq. 1})$$

^1H and $^{13}\text{C}\{^1\text{H}\}$ Nuclear Magnetic Resonance (NMR) spectra (Advance III HD spectrometer, Bruker Corporation, Billerica, Massachusetts, USA) were recorded at 300 MHz for ^1H and 75 MHz for ^{13}C using deuterated dimethylsulfoxide (DMSO- d_6) at 25 °C as the solvent.

Tensile properties analysis was conducted using samples measuring $5 \times 9.44 \times 0.122$ mm, employing a Shimadzu EZ Test Compact Table- Top Universal Tester at a speed of 1 mm/min^{-1} (EZ Test EZ-LX, Shimadzu, Kyoto, Japan).

2.3. Synthesis of the monomer N-(4-(phenylamino)phenyl)methacrylamide (SA-R1036)

In a 250 mL round-bottom pressure flask equipped with a magnetic stirrer, 2.08 g (11.29 mmol) of *N*-phenyl-*p*-phenylenediamine was dissolved in 180 mL of anhydrous diethyl ether. The mixture was stirred at room temperature (approximately 22 °C) to ensure complete dissolution and homogeneous mixing. Subsequently, 1.73 g (11.29 mmol, 1.68 mL) of methacrylic anhydride was added dropwise over 10 minutes to the stirred solution under ambient conditions. After the addition was complete, the reaction mixture was sealed and allowed to stir continuously overnight (approximately 16 hours) at room temperature to ensure full conversion.

Following the reaction, the solvent was removed under reduced pressure using a rotary evaporator at 40 °C until dryness. To eliminate any residual solvent traces, a stream of dry air was passed through the flask containing the solid residue for 15 minutes. The crude product was then purified by washing with

hexane (3×20 mL) to remove unreacted starting materials and impurities. The solid monomer was collected by filtration and dried under vacuum at room temperature for 12 hours.

The purified product was obtained as a solid with a yield of 70%. The chemical structure and purity of SA-R1036 were confirmed by spectroscopic analysis; full characterization details are provided in the Supplementary Information (SI-Section S1, Figure S1).

2.4. Synthesis of the smart polymer containing DPPH motifs

The synthesis of the sensor polymer was carried out in two stages. First, the polymerization of the different monomers was performed to obtain the material in film form, which we have termed "F." Second, a series of chemical reactions were carried out in the solid phase on the material F to obtain the smart material F_{DPPH} .

For the preparation of the material, the procedure described in previous publications^{10,13,16} was followed. It involves a bulk polymerization of the three selected monomers (VP, 49.75 mol%; MMA, 49.75 mol%; SA-R1036, 0.5 mol%), along with the crosslinking agent (0.1 mol% of the total monomers) and the thermal radical initiator AIBN (1% by weight of the total monomers). The resulting solution was injected into a mold (100 μm thickness). Oxygen-free polymerization was carried out at 50 °C overnight. The solid film was then demolded and washed with distilled water.

Once the material F was prepared, four solid-phase reactions were carried out on it, mimicking the DPPH synthesis reported in the literature^{17–21} (see supporting video), which is schematically summarized in Figure 1. First, the material F was immersed in a solution containing 250 mL of distilled water, 25 mL of HCl (37%), and 1 g of NaNO_2 , and kept submerged for 90 minutes at room temperature. In this reaction step, a diazonium salt is formed on the secondary amine of the SA-R1036 motifs present in the polymer. The material was removed from the solution without being washed; instead, the surface droplets of the film were gently wiped off with paper towels, and the film was immersed in a solution

of 4 g of $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 mL of HCl (37%) for 1 hour at room temperature. After this process, the material was washed with HCl (37%) three times (immersing the film for 15 minutes in each wash), then with distilled water (one 15-minute wash), a saturated NaHCO_3 solution (one 15-minute wash), and finally with distilled water again (three 15-minute washes), resulting in the material F1, containing phenyl hydrazine motifs.

The second reaction step is carried out in toluene as the solvent, so the material (which was initially immersed in water) was conditioned first in methanol and then in toluene. Subsequently, the material is immersed for 30 minutes at room temperature in a solution of 50 mL of toluene and 500 mg of 2-chloro-1,3,5-trinitrobenzene (synthesized according to the procedure described by Ismaili et al.²²; complete characterization in SI-Section S2, Figure S2). The material is then washed with toluene to remove any remaining 2-chloro-1,3,5-trinitrobenzene, resulting in material F2, which contains the motifs shown in Figure 1.

In the third reaction step, the material immersed in toluene is conditioned in dichloromethane. Meanwhile, a solution of 1 g of benzoyl peroxide in 50 mL of dichloromethane is dried with NaSO_4 (desiccant), and F2 is immersed in this solution overnight, protected from light with aluminum foil. Finally, the material containing DPPH motifs (F_{DPPH}) is washed with dichloromethane (three times for 15 minutes), methanol (once for 15 minutes), and water (once for 15 minutes).

The material is currently punched into 8 mm diameter discs using a punch and stored in airtight bags (zip bags). Alternatively, it can be stored in a mixture of methanol and distilled water in a 90:10 ratio, respectively. The characterization of the materials (F, F2, F3, and F_{DPPH}) is provided in the SI-Section S3, Figures S3-S6.

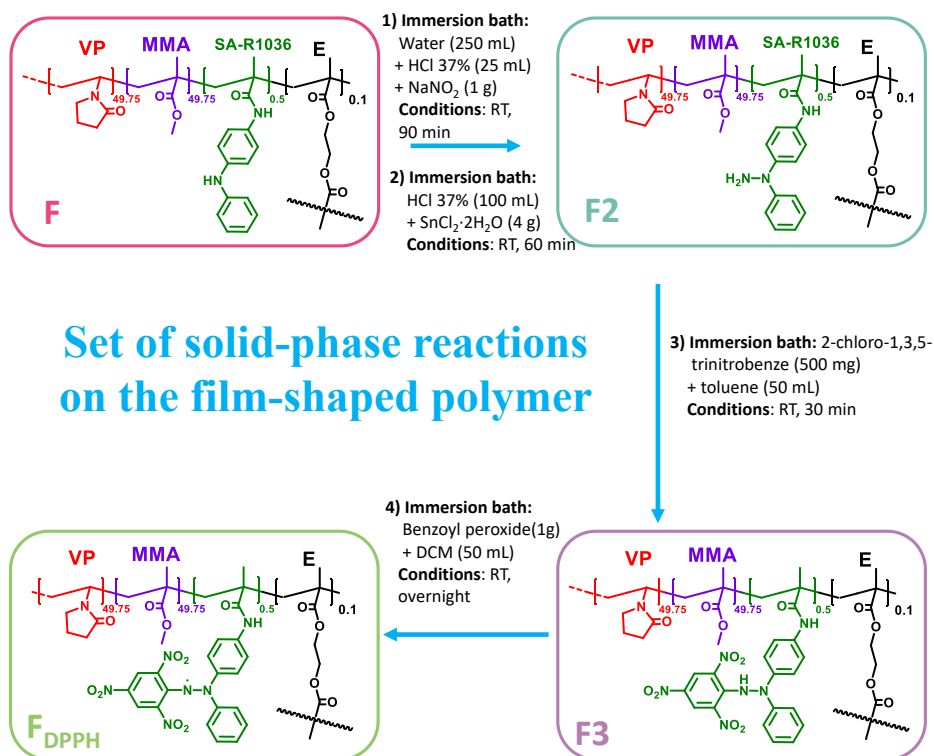


Figure 1. Schematic representation of the solid-phase reactions performed on the film-type polymeric material "F." In the first reaction step, the diphenylamine motifs undergo a benzene diazonium salt formation process, followed by reduction with tin chloride to form hydrazine. In the second step, an aromatic nucleophilic substitution occurs on 2-chloro-1,3,5-trinitrobenzene. Finally, the material is treated with benzoyl peroxide to obtain the DPPH motifs anchored onto the polymeric material.

2.5. Measurement of antioxidant capacity using the DPPH method

The DPPH assay was conducted following the protocol described by ²³ with slight modifications to adapt to our samples. A fresh stock solution of DPPH was prepared daily by dissolving 40 mg of DPPH radical powder in 100 mL of analytical grade methanol. This solution was stored in amber glassware to protect it from light degradation.

To prepare the working DPPH solution, the stock solution was diluted with methanol until the absorbance at 525 nm, measured with a UV-Vis spectrophotometer (UV-6300 PC, VWR, Leuven, Belgium), fell within the range

of 0.70 to 0.80. This absorbance range was selected to ensure optimal sensitivity and linearity of the assay.

For the assay, 980 μL of the working DPPH solution was mixed with 20 μL of the sample under study. In this research, the samples corresponded to the liquid generated during the cooking process of ready-to-eat beetroots, which serves as an indicator of the antioxidant capacity of the beetroot.²⁴

The mixture was incubated at room temperature (approximately 22 °C) for 120 minutes in the dark to prevent photodegradation of the DPPH radical. After incubation, the absorbance at 525 nm was measured using a UV-Vis spectrophotometer, with methanol used as the blank.

The decrease in absorbance corresponds to the scavenging of DPPH radicals by antioxidants present in the samples. Quantification was performed using a calibration curve prepared with Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ranging from 2 to 20 $\mu\text{mol/L}$. Results are expressed as mmol equivalents of Trolox per liter (mmol eq Trolox/L).

All measurements were performed in triplicate, and results are reported as the mean $\pm$ standard deviation.

2.6. Colorimetric assays using F_{DPPH}

Discs of the polymer film with a diameter of 8 mm were immersed in a mixture composed of 1.8 mL of methanol, 0.16 mL of deionized water, and 0.04 mL of the corresponding cooking liquid sample. The immersion was conducted at room temperature (approximately 22 °C). For the main assay described in this study, the discs were left immersed overnight (~16 hours) to ensure that chemical equilibrium between the polymer and antioxidants in the sample was reached.

Additionally, kinetic response time assays were performed with immersion durations of 15, 30, 60, 120, 180, 240, and 360 minutes to evaluate the optimal reaction time. At the end of each specified immersion period, the disc was carefully removed from the vial using tweezers to avoid contamination or damage.

The discs were immediately photographed inside a retro-illumination box to ensure consistent and uniform lighting conditions. A Xiaomi Redmi Note 10S smartphone was used for image capture, maintaining fixed camera settings for focus, exposure, and white balance to guarantee comparability between measurements.¹⁰

The captured images were analyzed using the Colorimetric Titration application to extract colour parameters in two widely used digital colour spaces: RGB (red, green, blue) and HSV (hue, saturation, value).²⁵ Among the measured parameters, the hue (H) component of the HSV colour space was found to exhibit the strongest correlation with antioxidant capacity and was therefore selected for further quantitative analysis.

All assays were performed in triplicate to ensure reproducibility, and the average values with standard deviations are reported.

2.7. Detection and quantification limits (LOD & LOQ)

The limits of detection (LOD) and quantification (LOQ) for the polymeric sensing materials were determined using Trolox as the reference antioxidant compound. Four known concentrations of Trolox solutions (1, 10, 20, and 40 ppm) were prepared in methanol and reacted with the sensing materials following the procedure described in Section 3.3 (Approach 2).

After incubation and image capture of the polymer discs as outlined in Section 2.6, the hue (H) parameter values were extracted from the digital images using the Colorimetric Titration application. The calibration curve relating the H parameter to Trolox concentration was constructed focusing on the low concentration range to ensure sensitivity.

The LOD and LOQ were then calculated using Equations 2 and 3, respectively:

$$LOD = \frac{3.3 \times SD}{s} \quad (2)$$

$$LOQ = \frac{10 \times SD}{s} \quad (3)$$

where SD represents the standard deviation of the samples and s denotes the slope of the calibration curve in the low analyte concentration region.

All measurements were performed in triplicate, and the average values of SD and slope were used for the calculations to ensure statistical reliability.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 8 (GraphPad Software, San Diego, CA, USA). Prior to analysis, the normality of the dataset was assessed using the D'Agostino & Pearson test to ensure the applicability of parametric tests.

To evaluate the strength and significance of the relationship between antioxidant capacity values obtained by the conventional DPPH assay and the colour parameters extracted from images of the FDPPH polymer discs (in both RGB and HSV colour spaces), the Pearson product-moment correlation coefficient (r) was calculated.

A correlation coefficient value (r) close to +1 or -1 indicated a strong positive or negative linear relationship, respectively. Statistical significance was determined at a p -value threshold of less than 0.05. Additionally, the adjusted coefficient of determination (adjusted R^2) was considered to assess the goodness of fit of the correlation models, with values above 0.9 indicating a very strong correlation.

The analysis of the differences between Approach 1 and Approach 2 was performed using a two-tailed paired t -test ($p < 0.05$).

All analyses were conducted using triplicate measurements, and results are reported as mean $\pm$ standard deviation unless otherwise stated.

3. Results and discussion

3.1. Characterization of F_{DPPH} smart polymer

The F_{DPPH} material has a structure based on two main monomers: VP and MMA. These two monomers provide the material with processability and the necessary hydrophilicity to be used in an organic-aqueous medium. Specifically, vinylpyrrolidone imparts rigidity and hydrophilicity to the material, while MMA contributes hydrophobicity. This 50:50 mixture of the two monomers has been used in similar smart polymers within the context of detecting species of interest in various foods, such as Hg(II) in fish,¹⁰ polyphenols in wine,¹⁴ nitrite anions in meat,²⁵ betalain in beetroots,⁴ Zn(II) in pet food,²⁶ Cu(II) in grape must,¹³ or lactose in milk.¹⁶

It is challenging to translate the manageability of this type of smart materials into a numerical value, as it ultimately refers to the ease of handling in a specific solvent (or solvent mixture) and with a particular experimental procedure. However, there are some data that can provide an indication of whether the material is suitable for the intended application, beyond the experimental test itself.

Among other factors, the thermal characterization of the material through TGA and DSC indicates that the material is suitable for its intended application. For instance, its glass transition temperature is 136 °C (SI-Section S3-Figure S6d), which is appropriate considering that it is intended for use at room temperature, as above this temperature, the material would become rubbery and difficult to handle. On the other hand, the T_5 and T_{10} temperatures of the material are 334 °C and 348°C, respectively (SI-Section S3-Figure S6c). This suggests that the material could withstand high temperature washing processes without thermal degradation, which is relevant when considering potential washing cycles for material reuse. Furthermore, the good mechanical properties of the material are reflected in a Young's modulus of $1355 \pm 177 \text{ N}\cdot\text{mm}^{-2}$ and an elongation at break of $5.2 \pm 2.5 \%$. These values are consistent with those reported for similar

materials in the literature designed for comparable applications, reinforcing the suitability of the polymer for its intended use.^{27,28}

From a chemical characterization perspective, FTIR experiments were conducted. Unfortunately, they were not entirely conclusive regarding the chemical transformation of the materials F, F2, F3, and F_{DPPH}, as the sensitivity of this technique is not high enough to detect changes in the composition of the SA-R1036 motifs (SI-Section S3-Figure S6b). This monomer is included at 0.5 mol% in the film-shaped smart polymer, which is highly advantageous from a material cost perspective, but it poses a challenge when attempting to characterize structural changes at each stage of the solid-phase reaction. Therefore, the clearest signals in these IR spectra are those contributed by the commercial monomers VP and MMA, which make up 99.5 mol% of the material. A strong band at 1723 cm⁻¹ is attributed to the ester carbonyl (C=O) stretching of MMA, while the lactam carbonyl of VP appears at 1667 cm⁻¹. Due to their low concentration, the carbonyl bands from SA-R1036 and the crosslinker are negligible. Other characteristic absorptions include the C–H stretching in the 2830–3020 cm⁻¹ range from the polymer backbone. A broad band in the 3200–3600 cm⁻¹ region is also observed, due to residual moisture. The spectrum aligns well with the expected composition, confirming the structure of the polymer. No significant differences are observed in the IR spectrum of F_{DPPH} compared to the initial material, as the modified monomer's low percentage falls below the detection limit of FTIR.

Since it is a crosslinked material, it cannot be dissolved for NMR experiments. Even if it could be dissolved, we would encounter the same limitation: the sensitivity of NMR, which is not high enough to identify these structural changes.

One might think that it would have been more appropriate to synthesize the DPPH-derived monomer by incorporating a polymerizable group (vinyl group) and then carry out copolymerization with VP and MMA. However, this strategy is much less environmentally friendly, as it involves greater consumption of

reagents and solvents. Our proposed solid-phase reactions are much simpler and align better with green chemistry principles, such as prevention, atom economy, safer synthesis, and energy efficiency optimization, among others. Our preparation method is based on reactions previously published in the literature, but adapted to our material, both for hydrazine formation,^{20,21} nucleophilic aromatic substitution,¹⁸ and stabilized radical generation.¹⁹

To demonstrate that our material contains those DPPH motifs, we have studied its colorimetric behavior in the presence of different chemical reagents and compared it with the behavior reported in the literature for the discrete DPPH molecule. Figure 2a shows the colour changes in solution of the discrete DPPH molecule reported by Ionita,¹⁷ while Figure 2b displays the colour changes of F_{DPPH} when subjected to the same chemical processes. This includes the interconversion of hydrazine (yellow) into a free hydrazyl radical by oxidation (purple), or into a salt upon reaction with a base (brown). As can be seen in the images, the colour changes of our smart material match those reported for the discrete molecule, which allows us to assume that these DPPH motifs have been formed in the polymeric material. This interconversion assay also highlights a highly relevant finding: the smart polymer F_{DPPH} can be reused and restored to its initial form after exposure to radical-scavenging species, such as polyphenols.

3.2. Results of antioxidant capacity measurement of 23 beetroot samples using the DPPH method

Table 1 (column “DPPH Method”) shows the results obtained from the antioxidant capacity analysis using the DPPH method, expressed as mmol of Trolox equivalent per liter (mmol eq Trolox/L). A wide variability in the total antioxidant capacity of the 23 beetroots analyzed was observed. The DPPH values of the samples ranged from 4.71 ± 0.06 and 11.71 ± 0.19 mmol eq Trolox/L. Differences in the antioxidant properties of commercial beetroots have been linked to cultivar, production system, processing or storing conditions, among other factors.^{29–31} Our results suggest that the samples selected may therefore be representative of the variable functionality that can be found in this root.

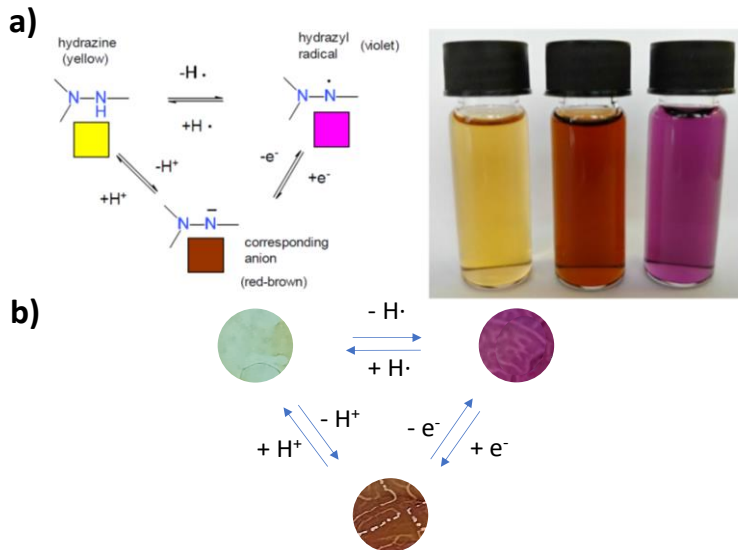


Figure 2. Interconversion of the different forms of the DPPH molecule: (a) in solution (Reproduced from Ionita, I from,¹⁷ under the Creative Commons CB 4.0 license) and (b) in the proposed FDPPH material. Hydrazine (yellow) transitions to a free hydrazyl radical by oxidation (purple) or into a salt upon reaction with a base (brown).

3.3. Results of colorimetric assays using F_{DPPH}

Two different approaches were employed to determine the antioxidant capacity of the 23 beetroot samples by photographing the F_{DPPH} discs and subsequently analyzing the RGB and HSV colour spaces. In the first approach, which we recommend, colour calibration was performed using the samples themselves, meaning no external standard was used for calibration; instead, the samples served as their own reference. In the second approach, we followed a more traditional protocol, calibrating the colour change of our material using Trolox. However, as we will explain below, this method does not account for the presence of other components and interferents within the food matrix, making it, in our opinion, a less advisable approach.

Approach 1

Figure 3a shows the photographs taken of the 8 mm diameter F_{DPPH} discs for each beetroot sample. The colour parameters obtained from the RGB and HSV colour spaces are available in SI-Section S4-Tables S1 and S2.

All colour parameters were plotted against the concentration data in mmol Trolox equivalents per liter (mmol eq Trolox/L) obtained from the traditional DPPH assay (Figure 3b). As a result of the analysis of the obtained data, we observed that the best correlation coefficient ($r=0.9524$), with the most significant p-value (<0.0001) and the best R^2 (0.9071) fit, corresponds to the H parameter (SI-Section S5, Table S3).

Once the calibration curve equation of the H parameter versus antioxidant capacity was obtained, the H values from each beet sample were simply input into the equation to determine the antioxidant capacity values, which are presented in Table 1 (column " F_{DPPH} Method (Calibration with beetroot samples) Approach 1"). This calibration curve is considered the most realistic, as it was constructed using actual beet samples, ensuring the presence of all potential interferents typical of this food matrix. In a hypothetical future analysis of the antioxidant capacity of a beet sample, the process would be as straightforward as inserting an F_{DPPH} disc into the sample (prepared according to Section 2.6), photographing the disc (using the same camera and backlit box that were used to construct the calibration), and applying the obtained H value to the cited equation.

From a practical perspective and in terms of knowledge transfer to the industry, a company would need to replicate the calibration using its own instrumentation. These procedures and data serve solely as a guideline for use and best practices.

Table 1. Antioxidant capacity results of the 23 beetroot samples tested. The table presents the data obtained using the reference DPPH method, as well as the results obtained with the proposed method based on the smart polymer F_{DPPH} and colour analysis, using both Approach 1 and Approach 2.

Beetroot Sample	DPPH Method		H parameter obtained from photographs		F_{DPPH} Method (Calibration with beetroot samples) Approach 1		F_{DPPH} Method (Calibration with trolox) Approach 2	
	Antioxidant Capacity (mmol eq Trolox/L)	SD	H	SD	Antioxidant Capacity (mmol eq Trolox/L)	SD	Antioxidant Capacity (mmol eq Trolox/L)	SD
A	9.18	0.23	4.35E-02	1.12E-02	8.68	1.28	8.66	1.27
B	9.58	0.09	4.44E-02	1.00E-02	8.79	1.14	8.77	1.14
C	4.71	0.06	8.33E-03	2.78E-03	4.69	0.32	4.67	0.31
D	8.33	0.05	4.72E-02	1.27E-02	9.10	1.45	9.08	1.44
E	8.63	0.05	4.35E-02	9.76E-03	8.68	1.11	8.66	1.11
F	7.57	0.11	2.78E-02	1.27E-02	6.90	1.45	6.88	1.44
G	10.45	0.13	6.30E-02	8.49E-03	10.89	0.96	10.87	0.96
H	10.69	0.55	6.11E-02	1.21E-02	10.68	1.38	10.66	1.37
I	6.68	0.08	1.67E-02	2.78E-03	5.63	0.32	5.62	0.31
J	6.80	0.11	2.78E-02	1.00E-02	6.90	1.14	6.88	1.14
K	7.13	0.04	3.06E-02	4.81E-03	7.21	0.55	7.19	0.55

Beetroot Sample	DPPH Method		H parameter obtained from photographs		F _{DPPH} Method (Calibration with beetroot samples) Approach 1		F _{DPPH} Method (Calibration with trolox) Approach 2	
	Antioxidant Capacity (mmol eq Trolox/L)	SD	H	SD	Antioxidant Capacity (mmol eq Trolox/L)	SD	Antioxidant Capacity (mmol eq Trolox/L)	SD
L	7.67	0.10	4.26E-02	1.85E-02	8.58	2.10	8.56	2.10
M	8.91	0.02	5.19E-02	1.43E-02	9.63	1.62	9.61	1.62
N	6.36	0.12	2.69E-02	8.93E-03	6.79	1.01	6.77	1.01
O	11.71	0.19	6.94E-02	1.21E-02	11.63	1.38	11.60	1.37
P	7.13	0.13	3.89E-02	1.44E-02	8.16	1.64	8.14	1.64
Q	7.96	0.06	2.87E-02	8.02E-03	7.00	0.91	6.98	0.91
R	8.46	0.08	3.98E-02	6.99E-03	8.26	0.79	8.24	0.79
S	5.52	0.02	1.02E-02	8.02E-03	4.90	0.91	4.88	0.91
T	6.11	0.05	1.57E-02	6.99E-03	5.53	0.79	5.51	0.79
U	11.52	0.16	6.94E-02	5.56E-03	11.63	0.63	11.60	0.63
V	6.30	0.06	2.96E-02	5.78E-03	7.11	0.66	7.09	0.66
W	5.73	0.06	2.13E-02	1.12E-02	6.16	1.28	6.14	1.27

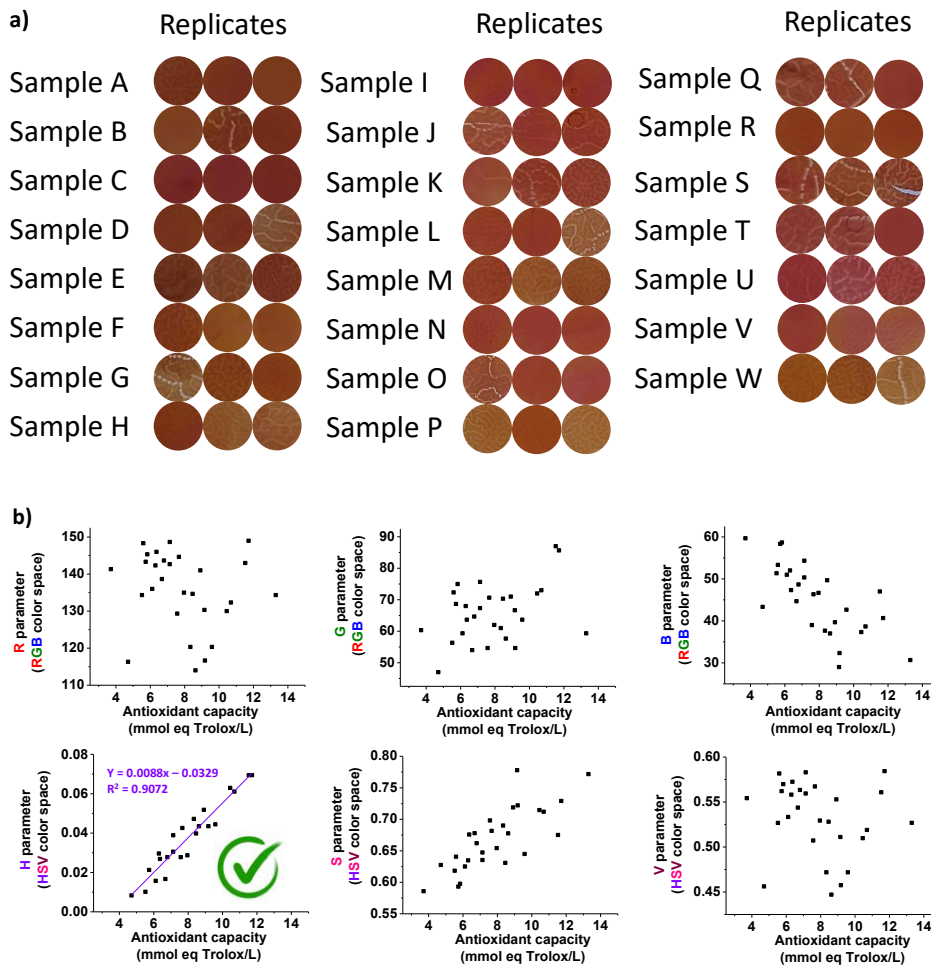


Figure 3. a) Photographs of the 8 mm diameter F_{DPPH} discs for each beetroot sample. Experimental conditions: The discs were immersed in a mixture of 1.8 mL of methanol, 0.16 mL of water, and 0.04 mL of the corresponding cooking liquid, at room temperature, overnight. b) Correlation between colour parameters (R, G, B from the RGB colour space and H, S, V from the HSV colour space) and antioxidant capacity (mmol eq Trolox/L) as determined by the traditional DPPH assay.

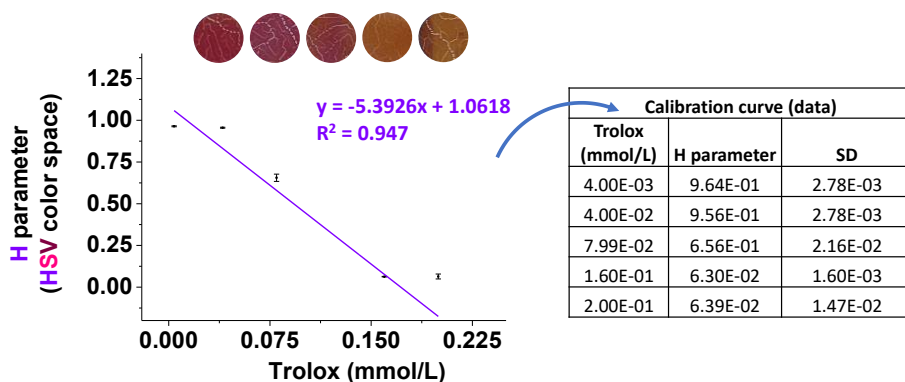


Figure 4. Calibration curve using Trolox as a standard. The image shows one of the three F_{DPPH} discs used at each concentration (4.00×10^{-3} , 4.00×10^{-2} , 7.99×10^{-2} , 1.60×10^{-1} , and 2.00×10^{-1} mmol Trolox/L). The discs were immersed in Trolox solutions in a methanol:water (90:10) medium overnight at room temperature, and subsequently photographed for the extraction of the H parameter from the HSV colour space.

The H parameter data obtained from the photographs of the F_{DPPH} discs for each beetroot sample were input into the calibration curve equation, with the corresponding dilution corrections applied. A slight deviation was observed compared to the data obtained using the traditional DPPH method. However, this deviation was consistent, allowing for a secondary correction to be applied to obtain the final data presented in Table 1 (column “ F_{DPPH} Method (Calibration with trolox) Approach 2”). We believe this deviation from the traditional method's results may be due to the presence of interferents in the food matrix, which is why we recommend Approach 1 over Approach 2.

Although the numerical values obtained with Approach 1 and Approach 2 appear closely similar at first glance, statistical analysis indicates a highly significant difference between them ($p < 0.0001$, paired t-test) (SI-Section S6, Table S4). This significance arises not from the magnitude of the difference, which is small in absolute terms (mean difference ≈ 0.02041), but rather from the remarkable consistency of that difference across the dataset. In nearly every case, Approach 1 yields slightly higher values than Approach 2. The paired t-test is particularly sensitive to such systematic deviations, detecting even small shifts when they are consistently in the same direction.

From a methodological standpoint, this difference is attributable to the nature of the calibration curves used. Approach 1 employs a calibration constructed using real food matrix samples, thereby incorporating the influence of matrix-specific interferences and better mimicking the complexity of actual sample conditions. In contrast, Approach 2 relies on a Trolox-based standard curve and extrapolates antioxidant values from that model, which does not account for the physicochemical interactions or interferences inherent to food matrices. Consequently, the statistically significant difference observed likely reflects the impact of matrix effects captured by Approach 1 but overlooked by the more idealized calibration in Approach 2.

3.4. Response time

The response time of materials designed for industrial applications is crucial for their success. From an academic perspective, we considered it appropriate for the tests with the smart polymer, in which the F_{DPPH} disc was immersed in the sample overnight, as this aligns with the standard procedures followed within our group and ensures that the reaction reaches equilibrium. However, in industry, where time is the most valuable resource, our method could be adapted to shorter times, assuming that the calibration (whether through Approach 1 or Approach 2) is carried out under the same conditions.

To this end, we immersed F_{DPPH} discs in a solution of Trolox (2.00×10^{-1} mM) and monitored the colour at different time intervals (15, 30, 60, 120, 180, 240, and 360 minutes). The results were highly revealing, as we observed that after 3 hours, the H parameter in the HSV colour space no longer showed any significant change (Figure 5). Additionally, between the 2- and 3-hour mark, a sharp colour shift occurred. This indicates that our method has a minimum induction time of several hours, during which antioxidant substances enter the smart polymer, interact with the DPPH motifs, and the reaction takes place. These results indicate that the optimal time for the assay would be 3 hours, as this is when equilibrium is reached.

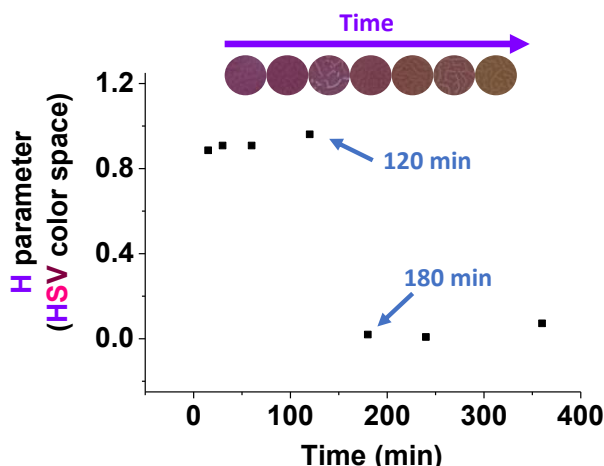


Figure 5. Study of the colour evolution of the smart polymer F_{DPPH} as a function of time when immersed in a solution of Trolox (2.00×10^{-1} mM). Experimental Conditions: Methanol:water (90:10) medium, room temperature.

3.5. Detection and Quantification Limits (LOD & LOQ)

The LOD and LOQ of the smart polymer F_{DPPH} were determined using Trolox as the reference standard. The values obtained for the LOD and LOQ were $6.32E-02$ and $1.91E-01$ mmol Trolox/L, respectively. These values are sufficiently low to ensure that the smart polymer can be effectively used in the proposed application. In fact, for the assays conducted, the sample was diluted at a 1:50 ratio, which further supports the system's capability to accurately and reliably detect and quantify antioxidant capacity.

4. Conclusions

This study demonstrates the potential of integrating advanced materials with smartphone-based analytical methods to transform antioxidant capacity assessment in the food industry. By covalently immobilizing DPPH molecules within a polymeric film, we developed a robust yet accessible sensor that simplifies the conventional DPPH assay into a portable, user-friendly device for rapid on-site quality control. Covalent attachment minimizes potential migration of free or mobile DPPH species. Previous studies using similar solid-phase synthesis materials have shown excellent migration assay performance and

compliance with European food contact standards, supporting material stability and suitability for food-related applications. Our conclusions rest on strong experimental evidence: a high correlation between the HSV hue parameter ($r > 0.96$, $p < 0.001$) and antioxidant capacity, successful calibration with real food matrices, an optimal 3-hour response time, and detection/quantification limits aligned with typical beetroot antioxidant levels. These results position the FDPPH sensor as a versatile platform extendable to other foods, encouraging wider use of smart polymer sensors in agri-food. Future work will focus on reducing assay time, expanding validation, and conducting toxicological and migration studies to confirm industrial safety.

Open Data

Open Data is available at <https://riubu.ubu.es/handle/10259/5684> under the name "UBU-Polymers Research Group 28032025".

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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assistant contract (UBU-08-B) funded by the Regional Government of Castilla y León.

Supplementary Material

Characterization of SA-R1036, the characterization of 2-chloro-1,3,5-trinitrobenzene, the characterization of polymers F, F2, F3, and F_{DPPH}; a table of color parameter data for digital color spaces (RGB and HSV); and a statistical analysis comparing Approach 1 and Approach 2. Video of solid-state reactions carried out on film-type material.

The Supplementary Material is available free of charge at:

<https://ars.els-cdn.com/content/image/1-s2.0-S0308814625033631-mmc2.pdf>

<https://ars.els-cdn.com/content/image/1-s2.0-S0308814625033631-mmc1.mp4>

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CHAPTER 3

Smart polymers in environmental remediation

This chapter focuses on the application of smart polymers in thin-film format for environmental remediation, particularly in polluted waters.

The first study presents a thin film bearing pendant NH_2 groups for the covalent immobilization of laccase using azo coupling, following the strategy introduced in the previous chapter. After optimizing and characterizing the immobilization process, the resulting reactive polymer is applied for the removal of bisphenol A (BPA) from contaminated waters. This study was published in *Environmental Technology & Innovation* (2025).

The second study, which derives from the first one, employs the same reactive thin film with immobilized laccase, but this time evaluates its performance in the degradation of indigo carmine dye in water, using natural phenolic mediators to enhance enzymatic oxidation. This article was published in *Results in Engineering* (2025).

The last study introduces a multifunctional smart polymer designed for nitrate management in drinking water. On the one hand, the material is capable of extracting nitrates due to the presence of NNZA monomers in the polymeric structure, which provide high anion-exchange capacity. On the other hand, the material enables detection of nitrate saturation through a fluorescence response, acting simultaneously as a reactive and sensory polymer. Moreover, this work incorporates a citizen science approach, in which hundreds of water samples from different localities were analyzed to determine nitrate concentrations. This research was published in *Environmental Technology & Innovation* (2025).

All three articles are reproduced in full in the following chapter. Each includes its own introduction that contextualizes the respective study, along with its materials, experimental procedures, results, and conclusions.

Development and characterization of a reusable laccase-polymer hybrid material for the removal of bisphenol A

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Development and characterization of a reusable laccase-polymer hybrid material for the removal of bisphenol A

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Abstract

Laccases are a group of versatile and green biocatalysts with high efficiency for the degradation of a wide variety of environmental pollutants. However, the feasibility of free laccase applications is hampered by enzyme inactivation due to exposure to denaturing conditions and very difficult recovery, which limits reuse and continuous process design, resulting in high costs. Enzyme immobilization technology is a very promising way of overcoming these drawbacks. In this work, laccases from two fungal sources, *Myceliophthora thermophila* and *Trametes versicolor*, were immobilized for the first time by covalent interaction (azo linkages) on a film-shaped polyacrylic material. Different parameters (enzyme dosage, reaction time, temperature) of the immobilization process were optimized, and the resultant immobilized laccases were characterized as a function of pH, temperature, and substrate concentrations (kinetic parameters) and compared with the free enzyme counterpart. The laccase-polymer hybrid (*M. thermophila* laccase) showed excellent operational and storage stability, retaining 89.8% of the initial activity after 15 cycles of reuse and 10 days of storage at 4 °C. Finally, the laccase-polymer films exhibited >90% depletion of bisphenol A in water by a dual adsorption-catalysis mechanism. The present work represents an attempt to develop a functional, stable, and cost-effective biocatalytic hybrid

material for application in the bioremediation of water and industrial effluents contaminated with BPA.

Keywords: acrylic polymer, biodegradation, bisphenol A, laccase, *Myceliophthora thermophila*, *Trametes versicolor*,

1. Introduction

Fungal laccases are enzymes of great interest in environmental biotechnology because they can degrade a broad range of xenobiotics, including phenols and derivatives, dyes, polycyclic aromatic hydrocarbons, pharmaceutically active compounds, personal care products, pesticides, plasticizers and others.^{1–3} These multi-copper oxidases do not require any additional/expensive substrate or cofactor, only molecular oxygen, which makes them very attractive compared to other enzyme systems.⁴

The efficiency of laccases for removing emerging organic contaminants from aqueous solutions has been demonstrated at a laboratory scale.^{5,6} However, the cost of the enzymes limits their large-scale application due to their lack of reusability and rapid denaturation when applied in free form. This problem can be mitigated by immobilizing the enzyme in/on different supports, such as hydrogels, nanoparticles, mesoporous silica, membranes, nanofibers, and polymeric materials.^{7–10}

For protein immobilization, synthetic polymers can offer some advantages such as molecular tailorability, inertness to microbial attack, biocompatibility, excellent processability and enormous available aggregation forms that can be achieved through different processing and self-assembling methods.¹¹ In this regard, several laccase-polymer systems have been developed yielding diverse outcomes.^{12–15} Recently, we have reported the synthesis of a highly manageable polyacrylic material for enzyme immobilization.¹⁶ This polymer prepared in a film shape has shown excellent reusability in both continuous and batch processes, offering significant practical advantages for biotechnological applications.

Bisphenol A (2, 2-bis(4-hydroxyphenyl)propane), is a widely manufactured chemical, largely used in polycarbonate plastics and epoxy resins for food packaging. This xenobiotic compound is an endocrine disruptor continuously released into the environment and has been declared a social, environmental and global issue. As a result, a great deal of work has been done over the last few years using immobilized laccases for their application in the degradation of this pollutant present in water and industrial effluents.^{17–20} However, the development of a stable and cost-effective biocatalyst with the possibility of recovery and reuse remains one of the major challenges for facing environmental biocatalysis, such as the treatment of wastewater containing BPA.

In this context, the present study aims to investigate the immobilization of two commercially available fungal laccases, from the thermophilic fungus *Myceliophthora thermophila* and the wood-rotting fungus *Trametes versicolor*, through covalent interaction with the new film-shaped acrylic polymer for stabilization and efficient reuse. To our knowledge, this approach has not been reported before. Optimization of the immobilization process and comparative characterization of the two fungal immobilized laccases in terms of optimum pH and temperature, kinetic parameters, and operational and storage stability were studied. Eventually, the efficacy of -polymer hybrids in the degradation of the emerging pollutant BPA was investigated.

2. Material and methods

2.1. Materials

1-vinyl-2-pyrrolidone (VP) (Acros Organic, 99%), methylmethacrylate (MMA) (Merck, 99%), 4-aminostyrene (SNH₂) (TCI, 98%), ethylene glycol dimethacrylate (E) (Aldrich, 98%), ethanol (VWR, 99.96%), methanol (VWR-Prolabo, 99.9%), hydrochloric acid (VWR, 37%) dimethylsulfoxide-*d*₆ (VWR, 99.8%), citric acid (VWR, ≥99.5%), disodium hydrogen phosphate anhydrous (VWR, 99%), sodium carbonate (Sigma-Aldrich, 99.9%), sodium azide (Alfa Aesar, 99%), sodium nitrite (Alfa Aesar, 98%), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) (Sigma-Aldrich, ≥98%), bisphenol A (BPA) (Aldrich, 99+%). Commercial laccases from *Myceliophthora thermophila* expressed in *Aspergillus oryzae* (≥1000 U/g), and from *Trametes versicolor* (≥0.5 U/mg) were purchased from Sigma-Aldrich. All the other reagents were analytical grade and used without further purification unless stated otherwise.

2.2. Synthesis of laccase-polyacrylic film

The acrylic polymer designed for laccase immobilization has previously been used as a support for β-galactosidase immobilization.¹⁶ This material stands out for its exceptional resistance to breakage, making it highly versatile and suitable for various applications without the need for delicate handling. Specifically, this film-shaped polymeric material comprises the following monomers: VP, MMA (as the main monomers), SNH₂ (in a smaller proportion as a functionalizing monomer), and E as the cross-linking agent. This acrylic polymer (F_{NH₂}) is synthesized by bulk radical polymerization at 60 °C overnight following the experimental procedure described by Vallejo-García et al. (2023)¹⁶. After the preparation of F_{NH₂}, three solid phase reactions were carried out (Fig. 1), briefly: (i) Formation of benzene diazonium salts through the NH₂ pendant groups, resulting in the material F_{N₂⁺}, (ii) Anchoring of the laccases into F_{N₂⁺} through the formation of diazo bonds, and (iii) blocking of the leftover free N₂⁺ groups with sodium azide, resulting in the laccase-polymer hybrid (FLac).

Laccase immobilization by azo coupling, the second step, was carried out in a 2.5 mL Eppendorf with 1 disc of $F_{N_2^+}$ and 0.75 mL of enzyme solution, in an orbital shaker at 25 °C. Different enzyme dosages of *M. thermophila* laccase in water (1, 5, 10, 20, 50, 100, and 150 U/mL) and treatment times (0.5, 1, 2, 6 and 24 h) were used to obtain the optimal immobilization conditions. It should be noted that the same enzyme solution could be used for a total of 6 discs with minimal reduction of laccase activity.

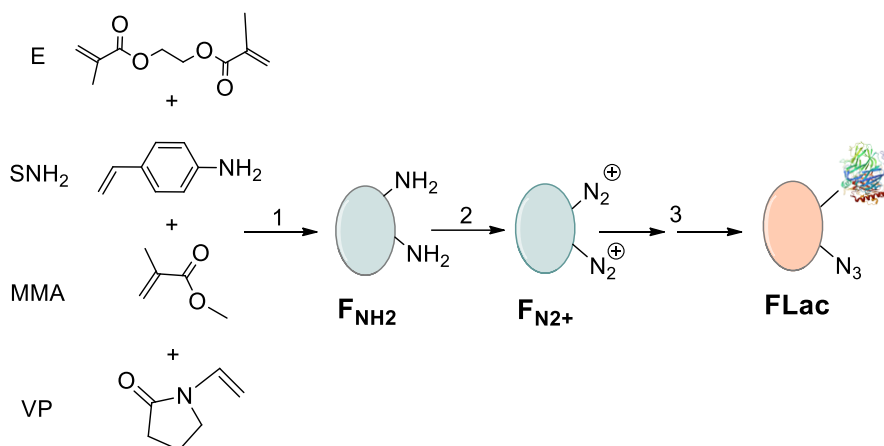


Fig. 1. A simplified schematic representation of the synthesis of laccase-polyacrylic film. The material is shown at different stages of the process: (1) Polymerization, F_{NH_2} (initial film with lateral aminostyrene groups), (2) diazotation, $F_{N_2^+}$ (film with lateral benzene diazonium groups), (3) azo coupling, FLac (film with anchored enzyme and free N_2^+ groups blocked with azide). VP (vinylpyrrolidone), MMA (methyl methacrylate), SNH_2 (aminostyrene), E (ethylene glycol dimethacrylate).

2.3. Laccase activity and protein content assays

The activity of laccase was determined spectrophotometrically using ABTS as substrate. Specifically, 0.1 mL of laccase solution or 1 FLac disc (6 mm diameter), 0.2 mL of 4 mM ABTS, 0.1 M NaOAc buffer at pH 4.0, in a total reaction volume of 2.0 mL, were incubated at 30 °C for 10 min in an orbital shaker at 130 rpm.

The reaction was stopped by adding 0.1 mL of 50% (w/v) TCA. Oxidation of ABTS was monitored in a microplate reader (PowerWave XS2, BioTek) with 96-well plates at 420 nm ($\epsilon=3.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$). Laccase activity was expressed as

$\mu\text{g ABTS}^{++}/\text{min.ml enzyme}$ or $\mu\text{g ABTS}^{++}/\text{min.cm}^2$ for the free and the immobilized enzyme, respectively. All assays were performed in triplicate including substrate blanks.

Protein content was determined using the bicinchoninic acid (BCA) assay, a reliable and suitable method for quantifying surface-bound protein.²¹ The immobilized enzyme (1 FLac disc of 6 mm diameter) was submerged in 1 mL of BCA reagent and 0.1 mL of distilled water and incubated for 30 min at 60 °C in an orbital shaker (130 rpm). Absorbance was read at 562 nm and the protein content was determined by comparison to a bovine albumin standard curve.

Specific activity was determined as $\mu\text{g ABTS}^{++}/\text{min.mg protein}$.

2.4. Biochemical characterization of free and immobilized laccase

2.4.1 Determination of kinetic parameters

To determine the kinetic parameters of free and immobilized laccases, enzymatic activity was measured for concentrations of ABTS ranging from 0.05 to 4 mM at 30 °C, according to the protocol described previously in section 2.3.

The Michaelis-Menten constant (K_m) and the maximum reaction rate (V_{max}) were calculated from Hanes-Woolf plot equation.

2.4.2. Optimal pH and temperature

To evaluate the optimum pH and temperature, the enzyme activity was determined at a pH range (2.0-7.0) of citrate-phosphate buffer and a wide temperature range (30-80 °C, 10 °C intervals). The relative activity of the free and immobilized laccases was calculated by considering the maximum activity as 100%.

2.5. Operational/storage stability

The stability of the immobilized laccases was evaluated in successive batch cycles of reuse and storage at 4 °C (2, 10, and 40 days) between batches. The experiment was performed in quintuplicate by incubating 1 FLac disc in 2 mL of 4 mM ABTS in acetate buffer (pH 4). Each cycle was performed at 30 °C for 10

min with orbital shaking. At the end of each cycle, the discs were washed three times with 4 mL of distilled water by vigorous vortexing, and maintaining them in water for 5 min between washes. In each batch, the FLac discs were reused in 5 consecutive cycles and then stored moist at 4 °C until reuse.

2.6. Determination of BPA removal efficiency

FLac discs (5 discs) were added to an aqueous solution of 80 mg/L BPA (5 mL) with orbital shaking (180 rpm) at 30 °C. Aliquots of 0.6 mL were taken to determine the BPA content after 0.5, 1, and 2 h of treatment. The discs were then separated from the reaction medium and washed with absolute ethanol for 30 min at 180 rpm to determine the adsorption of the phenolic compound on the immobilization support.

The content of BPA in the different samples was determined using a colorimetric assay for phenols under alkaline conditions, according to Modaressi et al. (2005)²². Reagents were added in the following order: 0.6 mL of BPA solution, 1.4 mL 0.1 M phosphate buffer at pH 8, and 0.1 mL 4-AAP (0.1 M). Potassium ferricyanide (0.1 M) was then added (0.1 mL) and allowed to stand for 10-15 min at room temperature. The absorbance was monitored at 506 nm, and the BPA concentration was determined from a standard curve²³ (Sadeghzadeh et al., 2020).

The removal efficiency was calculated by applying the following equation:

$$\text{Removal efficiency} = (C_{\text{BPA0}} - C_{\text{BPA}}) \times 100 / C_{\text{BPA0}}$$

where C_{BPA0} y C_{BPA} are the BPA concentrations (mg/L) before and after the treatment with the immobilized laccases, respectively.

Identification of biodegradation products of BPA was performed by GC-MS (6545 Q-TOF, Agilent) using a positive/negative electrospray ionization (ESI) source and 0.1% formic acid/methanol (30:70, v/v) as the mobile phase.

3. Results

3.1. Development of laccase-polyacrylic film (FLac) under optimized process parameters

To explore the optimal immobilization conditions, the effect of enzyme dosage (*M. thermophila* laccase) and treatment time on laccase activity in FLac were studied (Fig. 2). According to the results obtained, when the lowest enzyme concentration (1 U/mL) was used, the enzyme activity increased over time, but did not reach the activity levels of the other concentrations even after 24 h of treatment. At enzyme dosages of 5, 10, and 20 U/mL, laccase activity in the film increased up to 1-2 h and then decreased with immobilization time. In fact, the highest levels of active enzyme were observed during the first hour at a concentration of 10 and 20 U/mL, with a significant decrease in laccase activity of 32.5 and 54.1%, respectively, after 24 h. In particular, no differences were observed in the laccase activity of FLac obtained at enzyme concentrations of 20 or 10 U/mL when the immobilization time was 24 h.

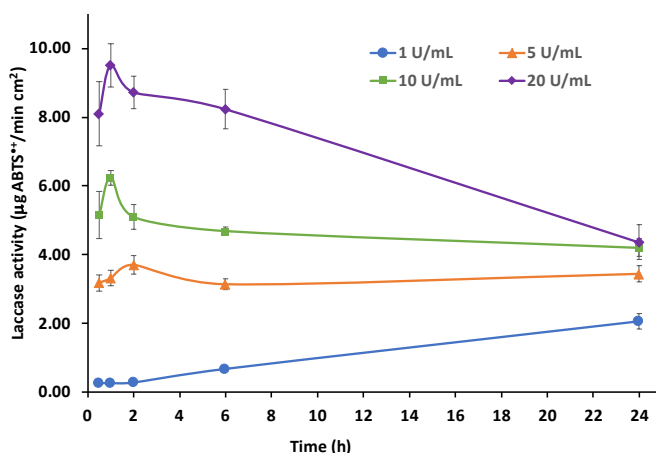


Fig. 2. Effect of the enzyme concentration and immobilization time on the activity of laccase-polymer hybrid (FLac). U, units of commercial laccase from *M. thermophila*.

To evaluate the efficiency of immobilization in terms of specific activity, the laccase activity and the amount of protein were determined in FLac obtained at increasing dosages of enzyme and for 1 h of treatment at 25 °C (Fig. 3). The results showed a progressive increase in enzyme activity and protein load on the films up to 20 U/mL. However, at higher enzyme concentrations (50, 100, and 150 U/mL), a significant decrease in laccase activity was observed with minimal changes in protein loading. The highest specific activities, 7.5, 7.8, and 6.3 $\mu\text{g ABTS}^{2+}/\text{min.mg protein}$, were obtained at 5, 10 and 20 U/mL, respectively.

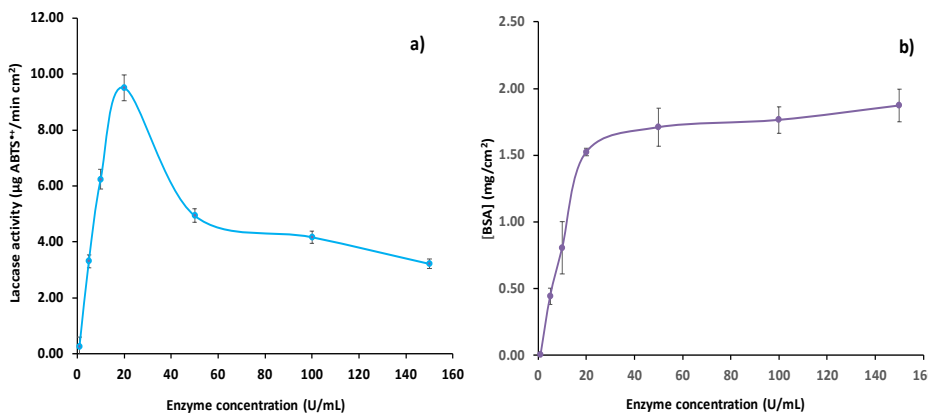


Fig. 3. Effect of the enzyme concentration on the laccase activity (a) and protein content (b) in the laccase-polymer hybrid (FLac). Immobilization time 1 h at 25 °C. U, units of commercial laccase from *M. thermophila*.

Based on the levels of active enzyme and the specific activity results in FLac, the enzyme dosages of 10 and 20 U/mL and 1 h of treatment were chosen to study the effect of the temperature on the immobilization process. According to the data obtained, increasing the temperature from 25 to 50 °C reduced the immobilized laccase activity by 32.7 and 35.8% for 10 and 20 U/mL, respectively.

According to the above, the optimal immobilization parameters for laccase immobilization on the acrylic polymer were 10 U/mL enzyme concentration, 1 h treatment, and 25 °C. These conditions were also used to immobilize the *T. versicolor* laccase in the comparative study of the two fungal laccases.

Laccase-polymer hybrids obtained with *T. versicolor* laccase under the optimized conditions, showed a 50.8% reduction in enzyme activity ($3.161 \pm 0.627 \mu\text{g ABTS}^{+}/\text{min.cm}^2$ versus $6.234 \pm 0.218 \mu\text{g ABTS}^{+}/\text{min.cm}^2$), and lower protein content ($0,327 \pm 0.056 \text{ mg/cm}^2$ versus $0,730 \pm 0.031 \text{ mg/cm}^2$) compared to FLac from *M. thermophila* laccase.

On the other hand, it was observed that the laccase-polyacrylic film became more intense in colour as the amount of protein anchored increased. To verify the relationship between colour and protein concentration, we used an optical technique (digital image correlation).^{24,25} FLac discs were photographed with a smartphone after dipping the $\text{F}_{\text{N}2+}$ discs in *M. thermophila* laccase solutions with concentrations ranging from 1 to 150 U/mL. The G parameter extracted from the digital images was represented against the protein concentration in the film. The trend was linear over this concentration range, as shown in Fig. 4. Specifically, the protein content of FLac (*T. versicolor* laccase) by resolving the value of the G parameter in the calibration equation (Fig. 4) was $0.362 \pm 0.013 \text{ mg/cm}^2$.

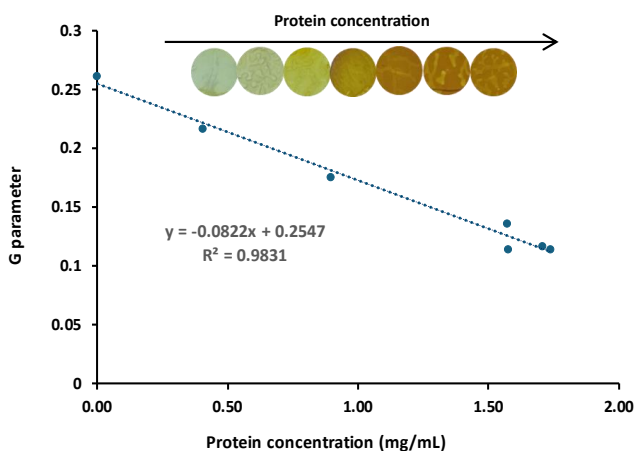


Fig. 4. Graphical representation of the G parameter from the photographed discs of laccase-polymer hybrid (*M. thermophila* laccase) versus protein concentration.

3.2. Biochemical characterization of fungal laccases immobilized on the acrylic polymer

A comparative study between the free and the immobilized enzyme for the laccases from the fungi *M. thermophila* and *T. versicolor*, was provided in terms of activity dependence on pH and temperature and kinetic behavior.

3.2.1. Determination of kinetic parameters

Soluble and immobilized laccases followed the Michaelis-Menten kinetic model but with different kinetic parameters. Table 1 shows comparative data for K_m and V_{max} , estimated by Hanes-Woolf plot using ABTS as substrate, for free and immobilized laccases. Although both fungal laccases showed similar affinity for the substrate (K_m), the free enzyme from *M. thermophila* was catalytically more effective against the ABTS substrate. The *T. versicolor* laccase immobilized on the film-shaped acrylic polymer showed a slightly higher K_m than the free counterpart, whereas no changes in K_m were observed for the *M. thermophila* laccase. For both FLac, the value of apparent V_{max} decreased compared to the soluble enzyme. In addition, immobilization increased the differences in catalytic efficiency between the fungal laccases, whereas the free *M. thermophila* laccase was 1.2 times more efficient than *T. versicolor* laccase in the immobilized form the efficiency increased by a factor of 2.3.

Table 1. Kinetic parameters for free and immobilized laccases (FLac) from *M. thermophila*^a and *T. versicolor*^b

Biocatalyst	V_{max} ($\mu\text{g ABTS}^{++} \text{ min}^{-1}$)	K_m (mM)	Efficiency(V_{max}/K_m)	R^2
Free enzyme ^a	74.07 ± 0.56	0.10	740.70	0.958
FLac ^a	6.00 ± 0.67	0.10	60.00	0.957
Free enzyme ^b	43.86 ± 0.10	0.07	626.57	0.953
FLac ^b	2.76 ± 0.28	0.11	25.09	0.950

3.2.2. Effect of pH and temperature on laccase activity

The effect of pH on the activity of the free and immobilized laccases was determined in the pH range of 2 to 7 (Fig. 5). In general, fungal laccases are acidic proteins and are quite active at pH 2.0-5.0.²⁶ As shown in Fig. 5, the two laccases were highly active in a narrow pH range between 2.0 and 4.0, with an optimal pH of 3 and 4 for laccase from *T. versicolor* and *M. thermophila*, respectively. Nevertheless, the pH profiles of the laccases were different, while the *M. thermophila* laccase had a bell-shaped profile, the *T. versicolor* laccase showed an ascending profile towards acidic pH values. It can also be observed that the pH profile of the immobilized laccase (*M. thermophila*) was slightly shifted towards acidic pH, with the optimum at pH 3 and retaining more than 40% of its maximum activity at pH 2, whereas the free enzyme retained only 14%. The shift of the pH activity optimum of immobilized laccase towards a more acidic region is not unusual and can also be found in the literature.^{27,28} The immobilized laccase (*T. versicolor*) showed a similar pH/activity curve to its free counterpart, with an optimum pH of 2.

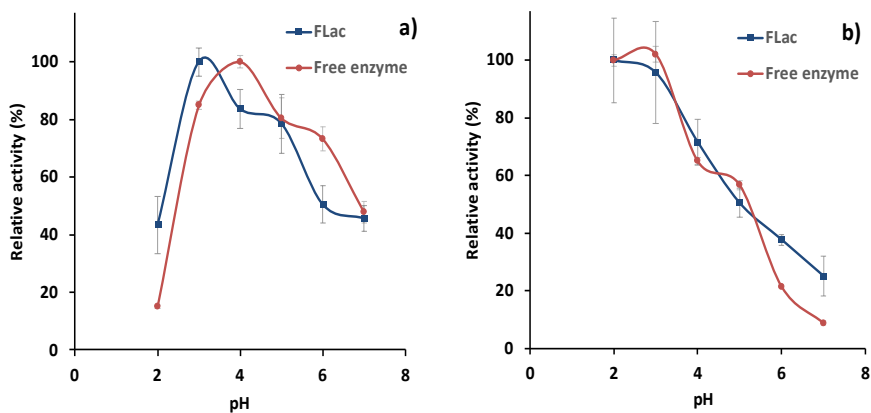


Fig. 5. Effect of pH on the free and immobilized laccase (FLac) from *M. Thermophila* (a) and *T. versicolor* (b).

However, the immobilized laccase retained 37.7 and 25.1% of the activity at pH 6 and 7, respectively, while the free laccase showed about 21.5 (pH 6) and 8.9% (pH 7). In this regard, *M. thermophila* laccase showed greater stability at these

pH values, with retention above 45% for both the free and the immobilized enzyme.

To investigate the effect of temperature on the free and immobilized enzyme, laccase activity was assayed at different temperatures from 20–80 °C (Fig. 6). The two fungal laccases, free or immobilized, maintained high relative activity (>60%) over a wide temperature range (40–80 °C). The optimum temperature for *T. versicolor* and *M. thermophila* laccase was 60 °C and 80 °C, respectively. The immobilized laccases showed no significant differences in the activity temperature curves compared to their free counterparts.

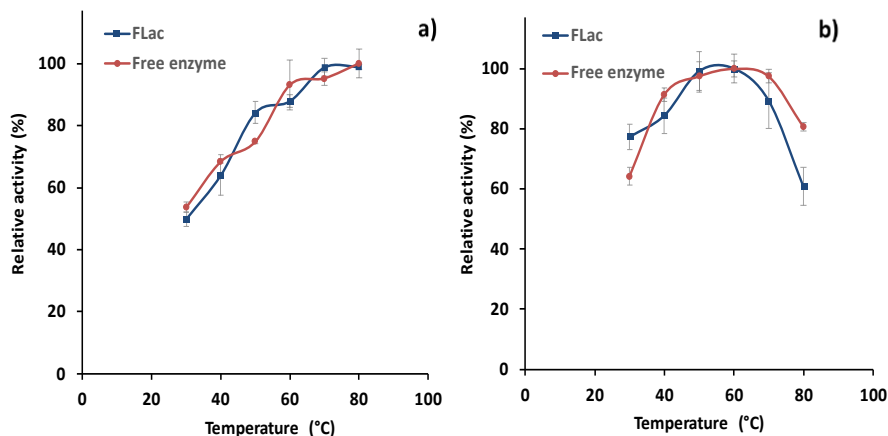


Fig. 6. Effect of temperature on the free and immobilized laccase (FLac) from *M. thermophila* (a) and *T. versicolor* (b).

3.3. Operational/storage stability

Several consecutive reaction cycles were carried out in batch experiments using the standard substrate ABTS to evaluate the operational stability of the immobilized laccases. The reusability of FLac was studied for up to five consecutive cycles before the samples were stored moist at 4 °C for subsequent recycling. FLac retained a residual activity of 100% (*M. thermophila* laccase) and 92.1% (*T. versicolor* laccase), after the first 5 cycles of reuse (Fig.7). For the industrial application of immobilized enzymes, it is also useful to have a better knowledge of their storage stability. As shown in Fig. 7, immobilized laccase from

M. thermophila and *T. versicolor* retained 89.9% and 69.5% of activity, respectively, after 10 days of storage at 4 °C and 15 cycles of reuse. After 40 days of storage and 20 reuse cycles, the FLac lost only between 31.2-38.3% of their activity.

3.4. Application of FLac for BPA removal

A gradual depletion of BPA by FLac was observed with increasing treatment time (Fig. 8). The removal efficiencies were between 34.0-37.9% after 30 min, 56-71% after 1 h, and 91.6-93.1% after 2 h. Therefore, both immobilized fungal laccases were shown to be effective in removing BPA. In addition, CG-MS analysis of the reaction media identified 3- or 2-methyl-2,3-dihydrobenzofuran, as a degradation product of BPA by laccase action.

To know the adsorption capacity of the immobilization support, the FLac discs used for BPA removal were washed with absolute alcohol (30 min at 180 rpm) and the concentration of phenolic compounds in the washes was evaluated. The adsorption percentage varied from $61.4 \pm 2.7\%$ (FLac discs with *M. thermophila* laccase) to $30.5 \pm 0.2\%$ (FLac discs with *T. versicolor* laccase). The phenolic compound 5,5'-bis-[1-(4-hydroxyphenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol, a degradation product of BPA, was identified in all ethanol washes together with 3- or 2-methyl-2,3-dihydrobenzofuran. In addition, BPA was also detected in the washes when FLac obtained with *M. thermophila* laccase was used. Interestingly, the immobilized laccases retained more than 93% of their initial activity after washing with absolute ethanol.

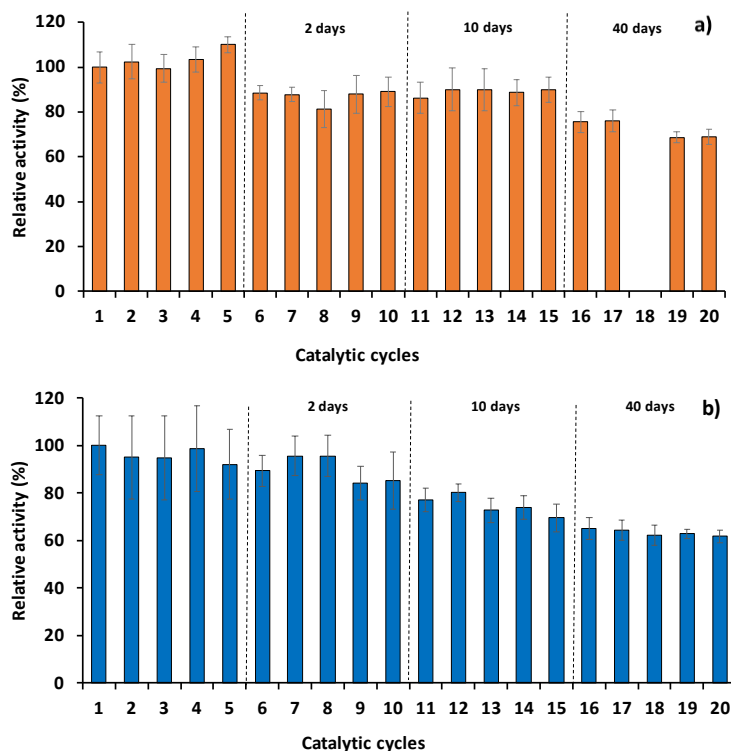


Fig. 7. Reusability and storage stability of laccase-polymer hybrid (FLac), (a) *M. thermophila* laccase, and (b) *T. versicolor* laccase. Recycling batches after 2, 10 and 40 days of storage at 4 °C. FLac activity in the first cycle was taken as a reference value of 100%. Values are the arithmetic mean $\pm$ standard deviation of five independent replicates.

4. Discussion

In this research, two fungal laccases were covalently immobilized on a film-shaped acrylic polymer through azo linkages. In order to optimize the immobilization process, the effect of enzyme concentration and immobilization time, parameters directly related to the final cost, were studied. It was observed that at the lowest enzyme dosages, all the protein offered was bound to the film, so that laccase activity increased with time to a maximum. In this sense, the loss of activity at higher enzyme concentrations could be explained by considering that as the treatment time increased, too many enzymes on the support surface could lead to a lack of space and steric hindrance and interactions between the

protein structures, resulting in limited mass transfer and a reduction in catalytic efficiency.²⁹ In addition, the functional groups on the surface of this film are limited, so that if there is an excess of enzyme, it cannot be anchored to the support by covalent bonding. The specific activity of the immobilized enzyme depends on its conformational flexibility, which can be altered at higher protein loads,⁷ which is in line with the higher specific activity obtained at enzyme concentrations of 10 U/mL as compared to 20 U/mL.

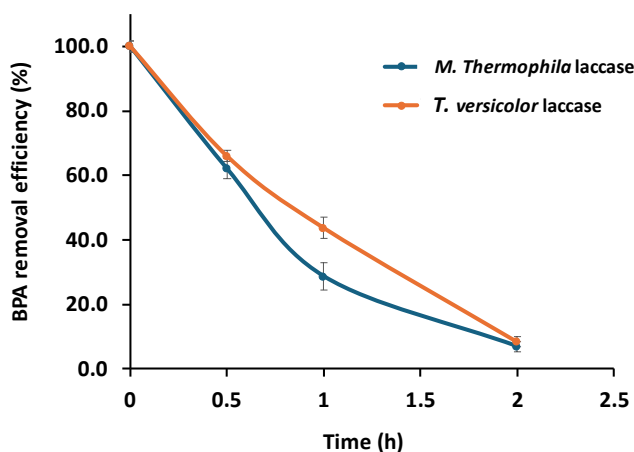


Fig. 8. Removal of BPA (80 mg.L⁻¹ BPA in deionized water, 25 °C) by laccase-polymer hybrid (FLac) *versus* time. Error bars are related to standard deviation of triplicate test.

On the other hand, the colour observed in the FLac discs was due to the azocoupling reactions with the phenol groups of the enzymatic protein, producing a compound with a strong yellow-orange color (an azo dye). In this research, a linear relationship between colour parameter “G” and the concentration of protein in the discs was described and compared to the BCA assay for quantification of protein in a surface. The results obtained by both procedures, with practically identical protein contents, confirmed their effectiveness for the direct quantification of protein immobilized on the film-shaped polymeric support. Furthermore, this property of the immobilization support could be used in further studies to develop a film-shaped colorimetric polymer sensor as an analytical tool for the determination of protein content in different samples.

Regarding the effect of temperature in the immobilization process, when an aryl diazonium salt is heated in the presence of water, the diazo group can be replaced via a phenyl cation.³⁰ This could explain the lower enzyme activity observed in the laccase-polymer hybrid obtained at 50 °C, as the coupling reactions with the aromatic functional groups of the protein were reduced.

Under the optimized immobilization conditions, FLac obtained with *M. thermophila* laccase showed higher catalytic activity than FLac obtained with *T. versicolor* laccase. These results can be related to the structural differences of laccases depending on their microbial source. Laccases from different sources share similar oxidation mechanisms and conserved copper-binding motifs, but show low sequence similarity and a broad oxidative spectrum.⁴ Similar results have been widely reported in the literature for laccases and many other enzymes.^{31–33} In a previously reported study, this polyacrylic film material also proved to be an efficient support for the immobilization of β -galactosidase from *Aspergillus oryzae*.¹⁶ However, this is the first time that this film-shaped material has been used to immobilize laccases.

Covalent immobilization techniques provide strong, stable enzyme attachment with reduced leaching and deactivation rates. However, during covalent immobilization, the native structure of the enzyme is usually affected due to the multipoint attachments and the enzyme activity is often reduced due to the rigorous treatment with toxic coupling reagents.⁸ Information about the possible interactions between the enzyme and its immobilization support can often be figured out by comparing the kinetic parameters of the immobilized enzyme and its free counterpart. The slow down of the reaction rate ($V_{\max}$) of FLac indicated that the immobilization process could lead to conformational changes in the enzyme, restrictions on the substrate transport, and steric constraints for biomolecular reactions.^{34,35} These effects appear to be more pronounced for *T. versicolor* laccase, which showed a significant decrease in activity after immobilization. Similar outcomes of lower $V_{\max}$ and catalytic efficacy after immobilization of laccases have been published.^{6,14,36,37} The K_m values for the

immobilized and free laccases were in the range of 0.07-0.11 mM, demonstrating that the binding affinity between the laccase-polymer hybrids and the substrate was similar to that of free laccase. The same behavior has also been reported by Vallejo-García et al. (2023),¹⁶ using this acrylic polymer to immobilize β -galactosidase and by other authors using different immobilization methods with laccases.^{38,39} On the contrary, some studies have reported higher and lower K_m values of immobilized laccases.^{37,40} Thus, the affinity of laccase to catalyze ABTS oxidation may or may not be altered by the immobilization procedure, depending on the specific characteristics of the enzyme and the immobilization method and support used.

It is well known that pH and temperature are important factors affecting the activity and stability of enzymes. The pH and temperature optima of laccases are very substrate-dependent and can vary considerably due to changes in the reaction caused by the oxygen, substrate, or the enzyme itself and its origin. The pH optima obtained in this study are consistent with the pH range of 3-5 reported in the literature for laccases using ABTS as a substrate.^{13,20} The bi-phasic pH/activity profile of *M. thermophila* laccase could reflect the possible combinatory contribution from the opposing effects of the pH-induced redox potential change (on both the T1 center and substrate) and the hydroxide anion inhibition (on the T2/T3 copper sites).⁴¹ In the case of *T. versicolor* laccase, the monotonic pH profile, when increasing the pH, could result from the involvement of hydroxide anion inhibition.⁴¹ In addition, possible changes in the dissociation and ionization state of the enzymes during the immobilization process could also explain the differences observed in the pH/activity curves.²⁹

On the other hand, the optimum temperature reported for laccases is in the range of 50-70 °C,⁴²⁻⁴⁴ which is also consistent with the results of this research. In general, the immobilized enzymes showed the same trend as their free counterparts. Thus, the differences observed in the temperature/activity curves between the two laccases would be related to their fungal origin and not

to the immobilization process. This behavior fits with the results reported by other researchers.⁴⁵

Operational and storage stability are significant advantages of immobilized enzymes from the point of view of industrial application. In this respect, satisfactory findings were obtained for the laccase-polymer hybrids. The acrylic polymer, as a protective support for laccases, maintained long-term recyclability and catalytic stability. The decrease in activity observed in FLac over successive cycles of discontinuous operation could be attributed to the partial inactivation of the enzyme due to mechanical damage during the handling of the immobilizates.^{23,35} This acrylic polymer was previously used as a support for the immobilization of β -galactosidase with similar results, showing a relative activity >99% after 10 cycles of reuse.¹⁶ Numerous studies have investigated the operational stability and reusability of laccase immobilized on a wide variety of supports, with very different results. For example, Sathishkumar et al. (2014)⁴⁶ immobilized laccase on cellulose nanofibers and observed 33% activity loss after 10 ABTS oxidation cycles. In a similar work, Zhao et al. (2023)⁴⁷ immobilized laccase on flexible ceramic nanofibrous membranes and reported 80% residual activity after 5 cycles. Kolak et al. (2023)¹³ also described a residual activity of 33.2% of laccase immobilized in an electrospun fiber material after 10 repeated cycles. In terms of storage stability, Zayed et al. (2024)²⁰ described 71-83% of the original activity of a laccase immobilized in novel polymeric support material after 30 days of storage, and Maryskova et al. (2019)⁹ showed that laccase immobilized in a polyamide-nanofiber membrane preserved 50% of the activity after 30 days at 4 °C.

It is worth noting that most of the research described in the literature, including that mentioned above, does not simultaneously investigate reusability and storage stability as in the present work.

BPA, a hazardous pollutant commonly found in wastewater, was selected as a model for a proof-of-concept experiment on the applicability of laccase-polymer hybrids in bioremediation. The results obtained are excellent compared

to recent research reported in the literature. For example, Xing et al. (2022)⁴⁸, using a membrane-enclosed laccase, reported 40.7% degradation of BPA in 4 h at 40 °C with an initial concentration of 0.1 mM BPA. Maryskova et al. (2022)⁹ were able to remove 90% of BPA in 20 h using laccase immobilized onto laminated poly(acrylic acid) nanofibers, and Lugo-Bueno et al. (2022)⁴⁹ reported 76% removal of BPA in 6 h using immobilized laccases assisted by electrochemical processes.

The biotransformation products identified in this study correspond to some of the compounds described in the literature for laccase-catalyzed BPA removal. For example, Xing et al. (2022)⁴⁸ proposed a possible degradation pathway of BPA by *T. versicolor* laccase, in which 3- or 2-methyl-2,3-dihydrobenzofuran were the result of electron loss and alkylation of 4-(2-hydroxypropan 2-yl) phenol, one of the first degradation products of BPA. In addition, an oxidative condensation pathway of BPA has also been proposed to form oligomers with C-C bonds, such as 5,5'-bis-[1-(4-hydroxy-phenyl)-1-methyl-ethyl]-biphenyl-2,2'-diol BPA dimer.⁴⁸

The results showed that the catalytic action of laccase-polymer hybrids, rather than its adsorptive action, played a leading role in the degradation process of BPA. Nevertheless, the combined action of efficient adsorption and enzymatic reaction improved the degradation efficiency of the pollutant. It should also be noted that the immobilization support, due to its adsorption capacity, also removed some of the BPA degradation products from the reaction medium. A proposed mechanism for this behavior assumes that BPA is adsorbed on free sites of the polymer, and after initiation of biodegradation by laccase, the occupied sites are liberated, and the sorption-biodegradation cycle can start again.⁵⁰ Other researchers have also reported this dual behavior of adsorption and degradation. For example, Yang et al. (2023)⁵¹ showed a total degradation efficiency of 67.6% for Lac@HOFs/hydrogel composite, in which the adsorption contribution of the support was 54.4%. Sadeghzadeh et al. (2020)²³ described adsorption of 7.5 ppm of the initial 60 ppm by magnetic cross-linked laccase

aggregates from *T. hirsuta*, and Naghadi et al. (2017)⁵⁰ reported a biodegradation contribution of more than 45%, while adsorption accounted for <30% of the total removal.

5. Conclusions

In this study, a durable, film-shaped, and easy-to-handle polymer was produced and successfully used as support for the covalent immobilization of two fungal laccases while preserving their biocatalytic activity. Analysis of the biochemical properties of the laccase-polymer hybrids (FLac) demonstrated their potential application over a wide range of pH (2-7) and temperature (30-80 °C), depending on the microbial source of the enzyme. This suggests that these biocatalytic films could be selected for application in a wide range of environmental conditions.

To evaluate the benefits of a biocatalytic system, its operational and storage stability are important factors in determining the processing costs. In particular, this immobilization procedure ensured both repeated use, with over 89-70% of initial activity retained after 15 ABTS catalytic cycles and storage stability at 4 °C (at least 40 days after repeated use). Taking this into account, the cost of 1 g of FLac (1 g of material corresponds to 400 discs) was estimated at 0.07 € (*M. thermophila* laccase) or 0.60 € (*T. versicolor* laccase), demonstrating its cost-effectiveness, especially when using the *M. thermophila* enzyme.

On the other hand, the FLac system achieved very efficient removal of bisphenol A (removal rates all above 90% in 2 h) through an adsorption-catalysis process, with an initial concentration of 80 ppm.

In conclusion, the present findings suggest that these laccase-polymer hybrids are a potentially useful tool in processes for the removal of BPA. Additionally, this work can be considered new step in the knowledge and application of the bioremediation in eliminating emerging organic contaminants in aqueous solutions

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The raw or processed data necessary to replicate these results are available at <https://riubu.ubu.es/handle/10259/5684> under the name “UBU-Polymers Research Group 12122024”.

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Supplementary Material

SEM images and element distribution (EDS).

The Supplementary Material is available free of charge at:

<https://www.sciencedirect.com/science/article/pii/S2352186425000793?via%3Dihub>

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*Natural mediators for indigo carmine dye removal with immobilized laccase
in polyacrylic films*

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Natural mediators for indigo carmine dye removal with immobilized laccase in polyacrylic films

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Abstract

This work presents a new polymeric material in the form of a film (F_{LAC}) containing immobilized laccase enzyme through diazo bonds, designed for the degradation of indigo carmine dye, which is highly toxic and commonly found in wastewater from the textile industry. The immobilization of the enzyme in the film was characterized by SEM, FT-IR, DSC, TGA and EDXS. The degradation of the dye by F_{LAC} initiates in the presence of a mediator due to the high redox potential of the dye. Six natural mediators (ferulic acid, syringaldazine, guaiacol, eugenol, thymol, and *p*-coumaric acid) were tested, and complete degradation of the dye was achieved in 180 minutes, with a mediator concentration of 1 ppm (syringaldazine) and a dye concentration of 10 ppm. A novelty in this study is the short exposure time of the dye-mediator solution to F_{LAC} (15 minutes) which allowed the degradation process to continue autonomously after the film was removed. Additionally, it was observed that the material was more effective in the presence of textile washing products, achieving over 99% degradation in 40 minutes, surpassing its efficacy in distilled water. Regarding reusability, the material retained more than 90% of its activity after five cycles of use and washing. Life Cycle Assessment (LCA) and Techno-Economic Analysis (TEA)

identified key levers for techno-economic and environmental viability: shorten cycle time (surfactant media), maximise re-use and per-cycle volume/concentration, implement solvent recovery, and energy decarbonization thereby outlining a roadmap to sustainable scale-up.

KEYWORDS: Laccase, enzyme immobilization, Indigo carmine, dyes bioremediation, smart-polymer, polymers, LCA, TEA.

1. Introduction

Classified as multicopper oxidases, laccases (EC 1.10.3.2) are broadly distributed among fungi, bacteria, plants, and certain insects. Their catalytic function involves oxidizing a wide spectrum of phenolic and non-phenolic substrates while reducing molecular oxygen to water. The catalytic mechanism relies on four copper atoms distributed across three binding sites: the type 1 copper site (T1), serving as the electron acceptor from the substrate, and a trinuclear cluster containing one type 2 (T2) and two type 3 (T3) coppers, which mediate oxygen reduction. Due to their high redox potential and broad substrate specificity, laccases play a crucial role in lignin degradation, stress response, and secondary metabolism. Their versatility has made them valuable tools in various biotechnological applications, including bioremediation, pulp and paper bleaching, wastewater treatment, biosensor development, and green chemistry.¹

The treatment of wastewater using laccase enzymes has been a subject of significant interest over the past decades.² This enzyme has been employed for the remediation of wastewater contaminated with antibiotics and pharmaceutical compounds,³ endocrine-disrupting phenolic compounds,⁴ and synthetic dyes.⁵ In this study, we specifically focus on its application in the degradation of dyes present in wastewater, with particular emphasis on indigo carmine (Acid Blue 74), a widely used dye in the textile industry, cosmetics, the food industry, and biomedicine.⁶ This dye is released into the environment through wastewater effluents, where it negatively impacts photosynthetic activity. and exhibits toxicity toward aquatic organisms.⁷ Furthermore, its complex

aromatic structure and high molecular stability pose challenges for biodegradation.⁸

Different methods have been used for the treatment of dyes like membrane technology, filtration,⁹ adsorption,^{10–12} or ion exchange,¹³ but most of them have shown some limitations like the high consumption of chemicals and energy, high costs of operation and maintenance and a high complexity.¹⁴ In previous publications from our group, we have based the treatment of water contaminated with these dyes on extraction procedures of this nature.¹² However, as the redundancy suggests, these methods are limited to extracting the dye from the aqueous medium in which it is present; they do not eliminate it, merely remove it. If the goal is the degradation of these compounds or a combination of extraction and degradation, enzymatic treatment with enzymes such as laccases should be employed.

The biodegradation of these dyes through the laccase enzyme emerges as an economically and technically viable alternative that can be integrated with other technologies. The enzymatic process can occur in one or two stages. For instance, if the target species to be degraded (such as a dye) has a low redox potential and is therefore suitable for the enzyme, the enzyme can react directly with the dye and degrade it. In certain situations, laccases cannot directly oxidize substrates either because their redox potential is too high or because they are not accessible to the enzyme's active site,¹⁵ requiring the presence of a mediator.¹⁶

Laccase mediators are low-molecular-weight compounds that undergo rapid oxidation by the enzyme and subsequently promote substrate degradation through indirect pathways. A wide range of mediators can be employed, including synthetic ones (e.g., ABTS, TEMPO, HBT) and natural ones (e.g., syringaldehyde, guaiacol, vanillin). Natural mediators, often phenolic compounds, are particularly attractive due to their low cost and non-toxic nature, leading to increasing interest in their application in recent years.^{8,17}

In addition to the use of mediators, another critical aspect to consider is the application of enzymes in their free form, which presents several limitations. These limitations include high costs in large-scale applications due to activity loss resulting from structural distortion under operating conditions, and difficult recovery and reuse.¹⁸ A widely adopted strategy to overcome these challenges involves the immobilization of enzymes onto solid supports, such as silica,¹⁹ hydrogels,²⁰ carbon nanotubes,²¹ iron oxide particles²² or acrylic polymers.^{23,24} Among the various types of supports used for laccase immobilization, synthetic polymers offer several advantages, including mechanical robustness, ease of handling, reusability, resistance to microbial degradation, biocompatibility, hydrophilicity, cost-effectiveness, and tuneable molecular structure and morphology.²⁴ Physical, chemical and combined methods have been investigated and developed using different supports. In contrast to physical approaches, chemical immobilization achieves enhanced stability as a result of the more robust bonds formed between the enzyme and the support.²⁵

In this context, we propose covalent immobilizing a commercial laccase from the fungus *Myceliophthora thermophila* onto a polyacrylic film support. The enzyme is immobilized chemically through azo bond formation mediated by the aniline functional groups of the polymeric support. This approach enhanced the degradation of indigo carmine with the assistance of natural phenolic mediators. This polymeric film is highly manageable and has been previously utilized by our research group with other enzymes, such as β -galactosidase (β -gal),²⁴ demonstrating excellent potential for efficient and user-friendly applications. A major advantage of this material is that it can be easily employed by simply immersing it in the indigo carmine contaminated medium and removing it upon completion of the process, eliminating the need for filtration steps for its recovery. Indeed, we have conducted experiments in which the material containing the immobilized catalyst is immersed for only a few minutes and then removed from the media. We have also tested the material in the presence of textile washing products, which significantly contributes to the accessibility and scalability of enzymatic degradation of this dye.

The proposed enzymatic degradation alternative is more environmentally friendly than the dye extraction techniques previously mentioned, as it not only permanently removes the dye, but also degrades it, enabling the direct reuse of the polymeric material. This also prevents the generation of secondary waste, making it a sustainable and efficient solution.

2. Experimental

2.1. Materials, Instrumentation and methods

Comprehensive information on the chemicals, solvents, reagents, instrumentation, and experimental procedures employed in this work can be found in the Supporting Information (SI-Section S1).

2.2. Design and synthesis of polymer material with immobilized laccase

The acrylic polymer designed for laccase immobilization has also been previously employed as a support for other enzymes.²⁴ The polymeric film developed demonstrates high resistance to mechanical stress, making it suitable for a broad range of applications without delicate handling. It consists of VP (hydrophilic structural monomer, 45 mol%), MMA (hydrophobic structural monomer, 45 mol%), and SNH2 (functionalizing monomer, 10 mol%, required for enzyme immobilization), with ethylene glycol dimethacrylate incorporated as a cross-linker at 0.1 mol% of the total material. Synthesis was carried out via bulk radical polymerization at 60 °C overnight, employing AIBN as the radical initiator.

Once FNH₂ was prepared, three sequential solid-phase reactions were performed to obtain the final laccase-immobilized material, as adapted from Vallejo-García et al. (2023) (Figure 1):²⁴ (i) formation of benzene diazonium salts at the -NH₂ pendant sites, producing FN₂⁺; (ii) covalent immobilization of laccases onto FN₂⁺ through diazo linkages, predominantly involving tyrosine and histidine residues,²⁶ and (iii) blocking of residual N₂⁺ groups with sodium azide, resulting in the FLAC film.

The main modification compared to the previously reported procedure concerns the reaction time in step (ii). Laccase immobilization via azo coupling was performed in 2.5 mL Eppendorf tubes containing one FN_2^+ disc and 0.75 mL of 10 U/mL enzyme solution, agitated on an orbital shaker at 25 °C for only 1 hour, rather than the overnight reaction previously applied for β -gal. This modification was introduced with the aim of optimizing the material preparation time. Another notable modification was made in step (iii), corresponding to the blocking of excess diazonium benzene groups with azide. This step was also optimized by varying the incubation times (5, 10, 20, 40, and 60 minutes). It is important to note that the same enzymatic solution can be used for up to six discs with minimal loss of laccase activity.

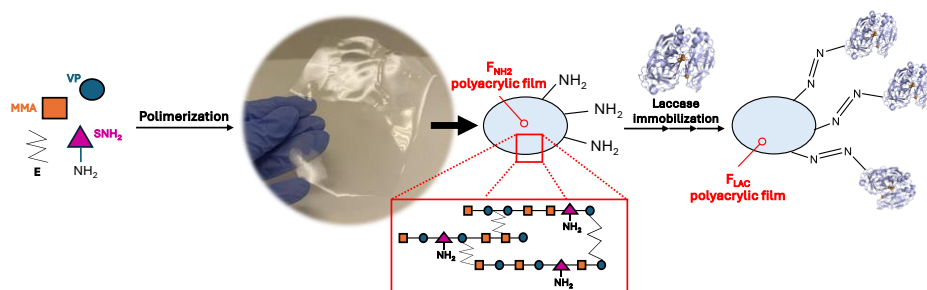


Figure 1. Simplified diagram of the synthesis of the laccase polymeric film. First, the polymeric film (F_{NH_2}) is synthesized through bulk radical polymerization of the monomers vinylpyrrolidone (VP, 45 mol%), methyl methacrylate (MMA, 45 mol%), 4-aminostyrene (SNH_2 , 10 mol%), and the cross-linking agent ethylene glycol dimethacrylate (E, 0.1 mol%). Then, laccase is immobilized through a series of intermediate steps, resulting in the final material (F_{LAC}).

2.3. Evaluation of immobilized laccase activity using a spectrophotometric method with ABTS

Immobilized laccase activity was determined via a spectrophotometric assay using ABTS. Each reaction contained a single F_{LAC} disc (6 mm diameter), 0.2 mL of 4 mM ABTS, and 0.1 M sodium acetate buffer (pH 4.0) in a total volume of 2.0 mL. The mixture was incubated at 30 °C on an orbital shaker at 130 rpm for 10 minutes. 0.1 mL of 50% (w/v) trichloroacetic acid (TCA) was added to finish the reaction. The oxidation of ABTS was measured at 420 nm ($\epsilon=3.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$)

using a microplate reader (PowerWave XS2, BioTek) and 96-well plates. Laccase activity was reported as $\mu\text{g ABTS}^{*+}/\text{min} \cdot \text{cm}^2$ for the immobilized enzyme. This assay was conducted to optimize the incubation times with azide, corresponding to step (iii) of the immobilization process described in the previous section. In addition, F_{LAC} was characterized as a function of pH, temperature, and substrate concentration (kinetic parameters) and compared with its free counterpart. These results were recently published by our research group.²⁷

2.4. Phenolic mediator screening for indigo carmine removal

The F_{LAC} polymeric material cannot directly degrade the indigo carmine dye and requires a mediator for the process to be successful. At the beginning of the study, several natural phenolic mediators (ferulic acid, *p*-coumaric acid, thymol, guaiacol, eugenol, and Syr) were tested for the degradation of indigo carmine. Initially, the mediators were dissolved in ethanol absolute to the desired concentration for solubility reasons. In UV-visible cuvettes, three F_{LAC} discs of 0.6 cm diameter were incubated in a final volume of 3 mL with 10% ethanol, a final indigo carmine concentration of 10 ppm, and varying concentrations of the different mediators. The F_{LAC} discs were kept in the solution throughout the entire process. For all the mentioned mediators, a final concentration of 10 ppm was used, except for Syr, due to solubility issues; for this mediator, concentrations of 0.5, 1, and 2 ppm were used. Guaiacol and eugenol were also tested at 1 and 50 ppm.

The reaction was conducted under orbital shaking at 190 rpm and 30°C and was monitored at different time intervals up to 300 minutes using UV/Vis spectrophotometer. To calculate dye degradation percentage, the following equation was employed:

$$\text{Indigo carmine degradation (\%)} = \frac{C_0 - C_F}{C_0} \times 100 ,$$

where C_0 and C_F , are the initial and final concentrations of the dye, calculated at $\lambda = 610\text{nm}$.

For the mediator and concentration that exhibited the highest dye degradation efficiency (Syr at 1 ppm), an additional study was conducted. In this experiment, the polymeric material was removed after a short exposure time (15 minutes), allowing the reaction to proceed autonomously once the dye degradation had been initiated.

2.5. Reusability of F_{LAC}

Using the brief exposure strategy to the F_{LAC} material described in Section 2.4, a reutilization study was conducted with the mediator Syr at a concentration of 1 ppm. For this, the 6 mm diameter F_{LAC} discs were extracted from the reaction medium after 15 minutes and washed three times for 5 minutes each with distilled water under orbital agitation at 190 rpm. After washing, the material was reincubated with fresh dye and mediator. This process was repeated for five successive cycles, and the dye degradation efficacy was assessed in each cycle.

2.6. Interference assays in the presence of textile washing products

Given that this material is designed for easy and straightforward use by non-specialized individuals, as well as in domestic environments, we decided to evaluate the material's efficiency in common washing waters that may contain laundry products such as fabric softeners or detergents. This essay was conducted following the procedure described in Section 2.4, using Syr as a mediator at a concentration of 1 ppm, while maintaining F_{LAC} in the medium throughout the entire reaction. In this case, 10 μ L of fabric softener and an additional 10 μ L of laundry detergent were added to the medium, using tap water instead of distilled water.

2.7. Life Cycle Assessment (LCA) Study

LCA was carried out in accordance with ISO 14040:2006 [28] utilizing SimaPro. Environmental impacts were quantified through the ReCiPe 2016 Endpoint (H) approach. The framework adhered to the standard LCA stages: (i) goal and scope definition, (ii) inventory analysis, (iii) impact assessment, and (iv) interpretation. Primary data were generated for the preparation and use of the F_{LAC} material and

for the reference system selected from the literature, while background processes such as energy supply, transport, and auxiliary materials were modeled using data sets from the Ecoinvent database. A comparative attributional LCA (ALCA) was performed to assess the environmental impacts associated with the use of two biocatalytic strategies for the degradation of 50 µg of indigo carmine dye.

The goal of this study is to compare the environmental impact of two alternative biocatalytic systems for the degradation of indigo carmine dye. The first is the novel film-shaped polymeric material containing laccase immobilized through diazo bonds (F_{LAC}), developed in this work, and the second is a reference solution based on laccase immobilized on chitosan–polyacrylic acid microspheres reported in the literature.²⁸ The comparison was established on the basis of an equivalent functional outcome, namely the degradation of a fixed amount of dye, and its ultimate purpose is to demonstrate that the material developed in this work was conceived with the intention of reducing environmental impacts. Although it is not feasible to include all immobilization strategies reported in the literature, the selected reference system was considered a relevant benchmark according to the criteria of the authors.

2.7.1. System Boundaries

The system boundaries of this study comprise all processes directly related to the preparation and use of the biocatalysts required to achieve the functional unit. This unit was defined as the degradation of 50 µg of indigo carmine dye in aqueous medium. This definition provides a common and measurable basis to evaluate both alternatives and ensures that their environmental performance is compared on the same functional outcome.

For both alternatives, this includes the synthesis of the polymeric support, the immobilization of the laccase enzyme, and the subsequent catalytic degradation process under the reported operational conditions. All chemical compounds were obtained from the Ecoinvent database; when an exact match was not available, a similar molecule was used as a proxy. Chitosan (75–85% deacetylated) and N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide

hydrochloride (EDC) were not considered in the modeling, as they could not be represented either in the database or through a suitable proxy.

2.7.2. Life Cycle inventory

Comprehensive life cycle inventory (LCI) data, covering all inputs and outputs associated with the F_{LAC} biocatalyst and the reference system, are reported in the Supporting Information (SI-Section S2, Tables S1–S2). These data form the basis for the subsequent comparative environmental assessment.

2.8. Techno-Economic Analysis (TEA) Study

The analysis was conducted at laboratory scale for a system comprising three circular discs (6 mm diameter, radius $r = 0.3$ cm) operated in $V = 3$ mL of indigo carmine (IC, 10 ppm) at 30 °C and 190 rpm. Two functional units were used: (i) area-based performance ($L \cdot m^{-2} \cdot cycle^{-1}$ and $L \cdot m^{-2} \cdot h^{-1}$), and (ii) specific cost per mass treated (€/g of IC removed). Unless explicitly stated, cost items associated with the mediator (e.g., syringaldazine, Syr), detergent/softener, operational utilities of the bench test, and waste handling are excluded, to isolate the contribution of the film.

Three kinetic scenarios were defined at constant volume and area (3 discs, 3 mL); differences therefore reflect kinetics (mediator dose and medium composition): (i) Scenario 1 (baseline): Syr 1 ppm, target removal at 180 min, (ii) Scenario 2 (higher mediator dose): Syr 2 ppm, target removal at 90 min, (iii) Scenario 3 (laundry-type matrix): water + softener + detergent with Syr 1 ppm, target removal at 40 min. More details about equations, assumptions, and step-by-step calculations underlying the TEA are reported in the SI-Section S3.

3. Results and discussion

3.1. Optimization of azide blocking times

As previously mentioned, our research group has utilized this material and immobilization method for the immobilization of other enzymes. One of the critical steps involves blocking the excess benzene diazonium groups after anchoring the enzyme, employing sodium azide. However, to reduce processing times and mitigate potential laccase inhibition caused by azide,²⁹ an optimization of the azide-blocking step was performed. As illustrated in Figure 2a, laccase activity decreases over time, reaching its minimum activity at 40 minutes and remaining stable until 60 minutes. Based on these observations, an azide incubation time of 5 minutes was selected, effectively optimizing the immobilization process without compromising laccase activity or the mechanical properties of the material.

3.2. Physicochemical characterization of F_{LAC}

Given that the material is designed for both industrial and domestic applications, it must exhibit good handling properties, which must be maintained under working conditions. These characteristics, perceivable by the end-user when handling the material, are closely related to its thermal properties. For instance, **thermogravimetric analysis (TGA)** (Figure 2b) reports T_5 and T_{10} values of 275 °C and 330 °C, respectively. Likewise, **differential scanning calorimetry (DSC)** (Figure 2c) reveals a glass transition temperature (T_g) of 130 °C. Both results confirm that the material meets the expected requirements under the intended operating conditions. Specifically, it does not undergo degradation or significant weight loss until temperatures exceed 250 °C and remains in a solid state below 130 °C (T_g). Regarding the immobilized enzyme, it is derived from a thermophilic organism with optimal activity at 80 °C. This ensures its proper functionality at typical temperatures used in the textile industry, where certain washing processes are conducted at 70 °C³⁰. It is worth noting that the DSC curve exhibits a prominent exothermic peak at approximately 200 °C, attributed to the

conversion of azide groups into nitrene groups. This transformation induces a highly exothermic curing process.³¹

The FTIR analysis (Figure 2d) of F_{LAC} revealed a broad band at 3300 cm⁻¹, attributed to the N–H stretching vibrations of the amide groups in laccase. Additionally, two C=O vibration peaks were observed at approximately 1720 and 1660 cm⁻¹, corresponding to the stretching vibrations of carbonyl groups. The peak at 1720 cm⁻¹ is attributed to the ester carbonyl (C=O) of MMA, while the peak at 1660 cm⁻¹ corresponds to the amide carbonyl (C=O) of VP. However, the C=O vibration of the amide I band in the protein may also contribute to the signal at 1660 cm⁻¹. Furthermore, two bands were identified around 650 and 550 cm⁻¹, which can be attributed to the metal centers in Cu–protein bonds. The band observed at 2900 cm⁻¹ corresponds to the C–H stretching vibrations of the aliphatic groups in the polymer monomers and the enzyme, while the band at 2100 cm⁻¹ is associated with the N=N stretching vibration of the azide group. These results confirm the successful binding of the enzyme to the material and are consistent with previous studies conducted by our research group, which employed similar polymeric films.^{12,24}

The WSP value obtained for the F_{LAC} material (89.4 ± 4%) is significantly different from that reported in previous studies for the same material but with a different immobilized enzyme (β-galactosidase (β-gal) enzyme, 139 ± 4%)²⁴. This difference could be attributed to variations in the two enzymes' structure, size, and hydrophilic nature. Laccase is a smaller enzyme (66.11 kDa) compared to β-gal (114 kDa) and has a GRAVY (General Hydropathy Average) value of -0.326, while β-gal has a GRAVY value of -0.479. Since more negative GRAVY values indicate greater hydrophilicity, this suggests that β-gal has a higher affinity for water, which would explain its higher WSP value. The GRAVY index values were calculated using the ProtParam tool from the ExPASy server.³²

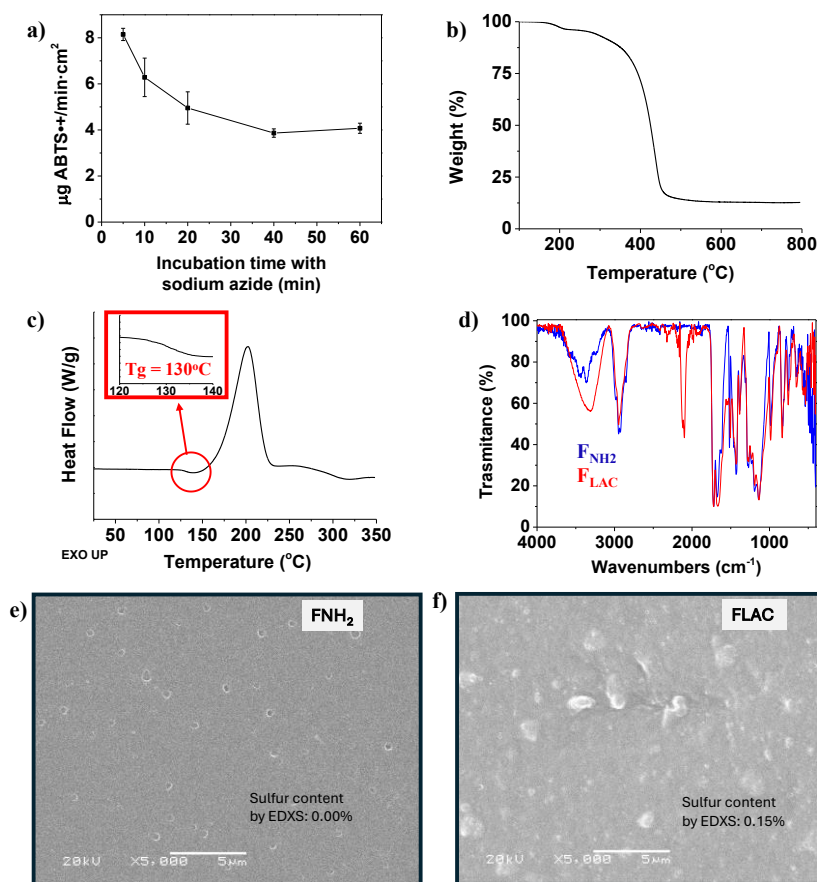


Figure 2. Physicochemical characterization of the F_{LAC} material. a) Optimization of azide blocking times. b) Thermogravimetric analysis performed in a nitrogen atmosphere at a heating rate of $10^{\circ}\text{C}/\text{min}$. c) DSC curve obtained with 10 mg of F_{LAC} at a heating rate of $20^{\circ}\text{C}/\text{min}$ in a nitrogen atmosphere. d) FT-IR spectrum. SEM micrographs of e) F_{NH_2} and f) F_{LAC} , showing sulfur content obtained through EDXS. The graph shows the ABTS activity ($\mu\text{g ABTS}^{+}/\text{min}\cdot\text{cm}^2$) of F_{LAC} materials blocked for different periods with sodium azide.

The SEM micrographs reveal increased roughness and the presence of particles in the enzyme-containing material (F_{LAC}), while the enzyme-free material (F_{NH_2}) only exhibits small defects in the form of pores, caused by the release of dissolved gases in the monomers during polymerization, as well as defects inherent to the used mold (Figures 2e and 2f, respectively). This increased roughness in the enzyme-containing material has been observed previously in

other studies.^{22,33} Qualitatively, this allows for the identification of the enzyme on the material's surface. However, quantitative analysis was performed using EDXS, specifically through sulfur analysis. The presence of this element in the F_{LAC} material (and its absence in the F_{NH2} material) is indicative of enzyme immobilization, due to the cysteine and methionine residues present in the protein sequence.³⁴ In the case of the laccase enzyme, the calculated sulfur content is 0.15%, a value consistent with that obtained from the EDXS analysis of the F_{LAC} material. This finding suggests that the enzyme is homogeneously distributed on the material's surface. Although this result supports the hypothesis that the enzyme is immobilized on F_{LAC}, it is essential to consider that EDXS analysis has a significant margin of error and provides information only about the material's surface, without assessing its internal composition.

3.3. Screening and evaluation of phenolic mediators for indigo carmine degradation

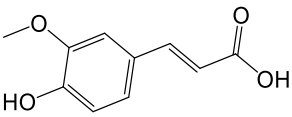
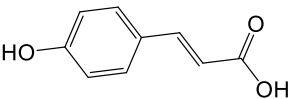
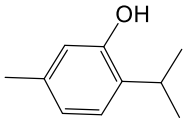
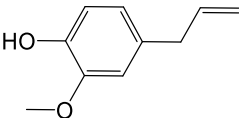
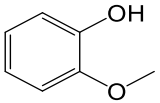
The catalytic efficiency and substrate versatility of laccase are increased by mediators, which operate predominantly via three pathways: (i) electron transfer, (ii) hydrogen atom transfer, and (iii) ionic mechanisms.^{35,36} Natural phenolic compounds selected for indigo carmine degradation by F_{LAC} follow a reaction mechanism based on the generation of phenoxy radicals via hydrogen atom transfer.^{37,38} The efficiency of these mediators in terms of speed and conversion yield depends on the redox potential and stability of the formed phenoxy radicals. The nature of the substituent groups on the benzene ring determines this potential.^{39,40} Table 1 shows the degradation percentages of indigo carmine at three different time points, using six different natural phenolic mediators, all at a final concentration of 10 ppm. The control film, without laccase, exhibited a slight reduction in dye concentration, likely due to the self-degradation of the dye or minor adsorption onto the film. However, this possible adsorption effect is negligible compared to the catalytic degradation achieved by the immobilized enzyme, confirming that the process is driven by enzymatic activity rather than adsorption. In mechanistic terms, the mediator first reaches the immobilized

laccase at the film surface to be oxidized at the enzyme's active site. Once oxidized, it diffuses back into the solution and reacts with Indigo Carmine, so the degradation occurs mainly in the bulk phase.^{39,41,42}

Ferulic acid, *p*-coumaric acid, and thymol demonstrated low degradation percentages, remaining near 10% after 300 minutes of reaction. In contrast, eugenol and guaiacol showed significantly higher degradation levels, reaching approximately 33% and 85%, respectively, in the same period. Notably, guaiacol achieved a remarkable 71% degradation in just 180 minutes. In the literature, different results can be found with these mediators and other dyes. For example, with *p*-coumaric acid and the dye Reactive Black, the degradation reached about 10% after 2 h at a concentration similar to that used in the present study, which is close to our findings. However, in that study, guaiacol did not lead to any noticeable decolorization.^{39,43} In contrast, another work reported that guaiacol enabled up to 80% decolorization of malachite green, although this required 24 h of reaction and a dye concentration of about 50 ppm. However, in that same study, *p*-coumaric acid and ferulic acid achieved much better results, exceeding 85% degradation after 6 h.⁴⁴ Evidently, the conditions across these studies are not fully comparable, as they used different laccases, dyes, concentrations, and reaction times. To our knowledge, the present study is the first to combine *M. thermophila* laccase with these specific mediators and this particular dye.

These results can be explained by the fact that laccase activity on phenols increases when electron-donating groups, like methoxy, are present on the benzene ring, as these groups facilitate the phenol group's ability to lose electrons. This helps explain the higher degradation rates observed for eugenol and guaiacol. In contrast, although ferulic acid contains electron-donating methoxy groups, its degradation rates are lower because the carboxyl group acts as an electron-withdrawing group, counteracting the effect of the methoxy groups.^{37,39}

Table 1. Degradation of indigo carmine using the laccase film and different natural phenolic mediators. Three 6 mm diameter F_{LAC} discs were introduced into 3 mL of dye solution (10 ppm) and mediator (10 ppm) in 90:10 water-ethanol solution. The percentage of dye degradation at different time points (60, 180, 300 min) is shown.

Structure	Mediators	Reaction time (min)		
		60	180	300
		Dye degradation (%)		
-	None (control)	0.81	0.44	1.58
	Ferulic acid	2.74	4.33	10.24
	<i>p</i> -Coumaric acid	2.29	6.10	8.43
	Thymol	2.47	4.65	7.39
	Eugenol	8.84	28.51	32.73
	Guaiacol	27.06	70.99	85.12

Due to solubility limitations, Syr could not be used at 10 ppm under the same reaction conditions (10% ethanol). Consequently, it was tested at lower concentrations (0.5, 1, and 2 ppm). Figure 3 shows the dye degradation achieved using these Syr concentrations over a 300-minute reaction period. Notably, even at the lowest concentration of 0.5 ppm, the results exceeded those obtained with the previously tested mediators at 10 ppm, achieving 31% degradation at 60 minutes, 76% at 180 minutes, and 92% at 300 minutes. At 2 ppm, the time required to reach degradation above 90% was significantly reduced, reaching this level in just 90 minutes. In comparison, at 1 ppm, the same degradation percentage was reached in 120 minutes. To achieve degradation rates above 99%, 1 ppm required 160 minutes, while 2 ppm achieved this in only 120 minutes. These results indicate that Syr is the most efficient mediator in this study. As mentioned earlier, this efficiency is attributed to the multiple methoxy groups in Syr structure (Figure 3), which not only facilitates the rapid oxidation of its phenolic groups but also enhances the stabilization of phenoxyl radicals. These findings are consistent with previous studies, where the most efficient mediators were syringaldehyde and acetosyringone, which share a very similar structure with Syr.⁴³ In other studies, the decolorization of indigo carmine in the presence of laccases from *Peniophora* sp. and *Bacillus velezensis* using syringaldehyde, showed efficiencies of 93% and 90%, after two and six hours, respectively.^{45,46}

Since eugenol and guaiacol were the mediators with the best results after Syr, a concentration of 1 ppm was used to compare their performance with Syr, and a higher concentration (50 ppm) was also tested (Table 2). At 1 ppm, eugenol showed very low degradation, reaching only about 11% after 300 minutes, while guaiacol exhibited better performance, achieving nearly 43% degradation in the same period. However, these results were significantly lower than those obtained with Syr at 1 ppm, which reached 50% degradation in just 60 minutes. To achieve nearly 99% dye degradation in 180 minutes with guaiacol, a concentration of 50 ppm was required. In contrast, under the same conditions, eugenol only reached approximately 31% degradation in 180 minutes and nearly 50% after 300 minutes. These results demonstrate that Syr at 1 ppm produced the best

outcomes with the lowest mediator consumption, making it the preferred option for subsequent experiments.

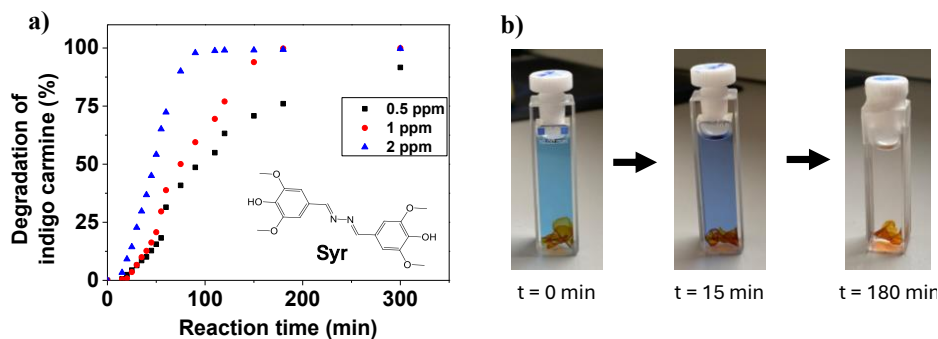


Figure 3. a) Indigo carmine degradation using the laccase film and Syringaldazine (Syr) at three different concentrations (0.5, 1 and 2 ppm). b) Images at 0, 15 and 180 min of the Indigo Carmine degradation process using Syr (1 ppm).

Since eugenol and guaiacol were the mediators with the best results after Syr, a concentration of 1 ppm was used to compare their performance with Syr, and a higher concentration (50 ppm) was also tested (Table 2). At 1 ppm, eugenol showed very low degradation, reaching only about 11% after 300 minutes, while guaiacol exhibited better performance, achieving nearly 43% degradation in the same period. However, these results were significantly lower than those obtained with Syr at 1 ppm, which reached 50% degradation in just 60 minutes. To achieve nearly 99% dye degradation in 180 minutes with guaiacol, a concentration of 50 ppm was required. In contrast, under the same conditions, eugenol only reached approximately 31% degradation in 180 minutes and nearly 50% after 300 minutes. These results demonstrate that Syr at 1 ppm produced the best outcomes with the lowest mediator consumption, making it the preferred option for subsequent experiments.

Table 2. Degradation of indigo carmine using F_{LAC} and the best natural phenolic mediators (Syr, eugenol, and guaiacol). A 6 mm diameter F_{LAC} disc was introduced into 3 mL of dye solution (10 ppm) and mediator (1 and 50 ppm) in 90:10 water-ethanol medium. The percentage of dye degradation at different time points (60, 180, 300 min) is shown.

		Reaction time (min)		
		60	180	300
	Concentration (ppm)	Dye degradation (%)		
Eugenol	1	7.51	10.09	11.20
	50	11.84	30.91	48.69
Guaiacol	1	7.48	20.87	42.46
	50	53.73	98.48	98.68
Syr	1	38.82	99.8	99.99

3.4. Dye degradation assays with short exposure to the F_{LAC} polymeric film.

One of the questions that arose during the research was the possibility that F_{LAC} acted solely as a degradation initiator, and that the system could then evolve independently. This scenario would involve an initial formation of the oxidized species of Syr by F_{LAC} , after which the presence of F_{LAC} in the media would become irrelevant. To investigate this hypothesis, once the peak absorbance of oxidized Syr was reached (15 min), as shown in Figure 4a⁴⁷, the film was removed ("film out") and the subsequent dye degradation was compared with that observed when the films remained present throughout the reaction ("film"). As shown in Figure 4b, the removal of the film resulted in a slower degradation rate; however, nearly 95% dye degradation was achieved after 400 minutes.

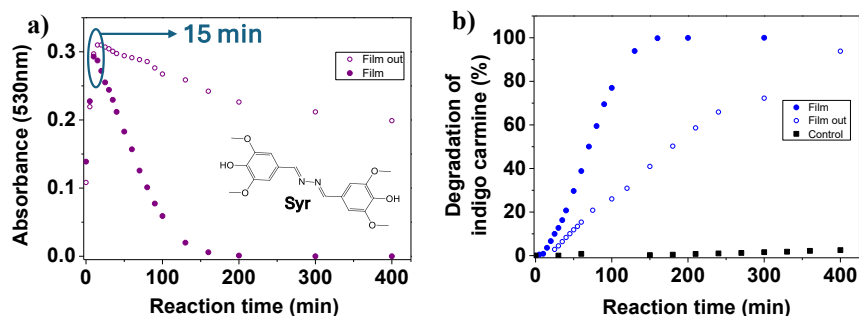


Figure 4. a) Evolution of the oxidized Syr species monitored at 530 nm over time. The laccase film with Syr at 1 ppm was tested using two different methods: (i) keeping the material in the reaction throughout the entire process ("film") or (ii) removing the material after 15 minutes and allowing the reaction to proceed without it ("film out"). b) Degradation of indigo carmine using the laccase film and Syr at 1 ppm using the methods (i) and (ii). A control with the material lacking the enzyme is also included for comparison. Experimental conditions: 30 °C; 190 rpm, 10 ppm indigo carmine, 1 ppm Syr, 90:10 water-ethanol medium.

It is worth mentioning that once the maximum absorbance of the oxidized form of Syr is reached, which acquires a pink coloration at 530 nm due to its oxidation by F_{LAC} , this absorbance gradually decreases over time (Figure 4a), with a much more pronounced decrease when the film remains in the reaction. This is because the enzyme continues to promote oxidation or secondary reactions occur. Laccase may be catalyzing further transformation of the oxidized Syr, leading to the formation of more stable dimers or polymers of phenoxyl radicals, thereby accelerating the disappearance of the pink coloration.⁴⁸

This phenomenon could also help explain why, when the F_{LAC} films are removed after 15 minutes, the degradation of indigo carmine appears slower compared to when F_{LAC} remains throughout the reaction. It is possible that the oxidized form of Syr is not the only species mediating the degradation; other reactive species, generated by subsequent reactions on the initial oxidized form, could also be playing a significant role in the process.

3.5. Reusability of F_{LAC} .

Reusability assays (Figure 5a) showed that the F_{LAC} material retained nearly 90% of its enzymatic activity after five rounds of use. By removing the materials after 15 minutes, it is possible to perform multiple dye treatment rounds in parallel, with only a 30-minute interval between each round (15 minutes of incubation + 15 minutes of washing). This strategy improves operational times, as it not only allows the reuse of the F_{LAC} material but also eliminates the need to keep it throughout the entire degradation process, limiting its presence to the first 15 minutes.

Previous studies with laccase-containing materials have reported similar results. Ruxandra-Leonties et al. reported a retention of 72% of the activity of laccase immobilized in chitosan-polyacrylic microspheres after five cycles of indigo carmine degradation.²⁸ Similarly, Liu et al. achieved approximately 80% activity retention under comparable conditions using laccase immobilized on silica nanoparticles.⁴⁹ In both cases, the laccase (or the materials containing it) remained present throughout the entire process. However, our study is, to the best of our knowledge, the first in which laccase-containing materials are removed right after the degradation reaction begins, allowing the process to continue autonomously in other vials while the system evolves without the presence of the material.

3.6 Impact of laundry detergents on indigo carmine degradation

Within the textile industry, Indigo Carmine is extensively utilized, with denim manufacturing representing its main area of application. During the dyeing process, washing with detergents and softeners is commonly applied. Since our material is designed for both domestic and industrial applications, we aimed to test it under conditions closely resembling real-world usage.⁶

As shown in Figure 5b, surprisingly, when the material acts in the presence of laundry detergent products, the dye degradation rate is significantly higher, reaching 100% in less than an hour. In contrast, in the distilled water

medium, this level of degradation is achieved in just over three hours. These results suggest an increase in the laccase activity in the presence of laundry products, which could be attributed to the surfactants present in these products. Surfactants may affect the conformation of laccase, making its active site more accessible to phenolic substrates. Furthermore, since Syr is poorly soluble in water, detergents could improve its solubility, making it more available to the enzyme.⁵⁰ This effect has also been reported in studies using other enzymes.⁵¹

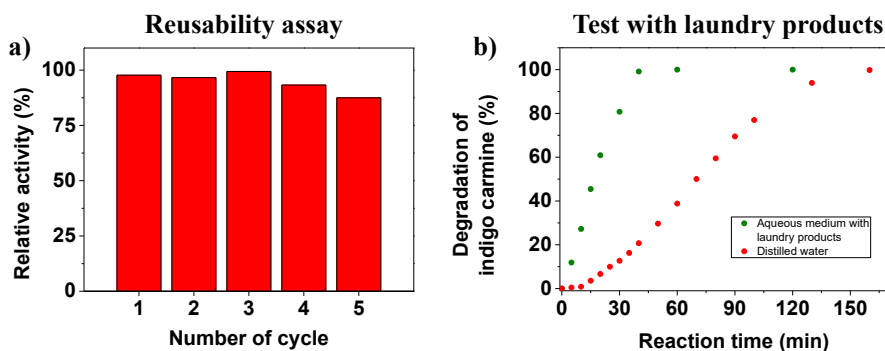


Figure 5. a) Reusability assay of the laccase film. Experimental conditions: 30°C, 190 rpm. Exposure time to F_{LAC} : 15 minutes; indigo carmine concentration: 10 ppm; Syr concentration: 1 ppm; total reaction time: 300 min. b) Degradation of indigo carmine using three F_{LAC} disks (6 mm diameter) in a simulated washing medium (3 mL distilled water, 10 mL fabric softener, 10 mL detergent), as well as in an aqueous medium (3 mL distilled water). Experimental conditions: 30°C, 190 rpm. Exposure time to F_{LAC} : throughout the entire degradation process; Syr concentration: 1 ppm; indigo carmine concentration: 10 ppm.

3.7. LCA Study

The LCA followed ISO 14040 using SimaPro and the ReCiPe 2016 Endpoint (H) method, with primary data for preparation and use of the diazo-bonded laccase film (F_{LAC}) and Ecoinvent datasets for background; proxies were used where exact matches were unavailable. The complete process trees for both systems (F_{LAC} and the literature benchmark with laccase on chitosan–polyacrylic acid microspheres)²⁸ are provided in the SI-Section S2, detailing the modelled flows of materials, energy and wastes to meet the functional unit (degradation of 50 µg indigo carmine in water). In the benchmark inventory, chitosan and EDC could

not be represented in Ecoinvent nor by suitable proxies; this limitation is disclosed and their inclusion would increase the benchmark footprint (biopolymer and activator production), making our comparison conservative in favour of the benchmark.

As shown in Figure 6, midpoint indicators were normalized against the benchmark (100%). In categories such as climate change (human, terrestrial, and freshwater), photochemical ozone formation, particulate matter, terrestrial acidification, freshwater eutrophication, and human/ecotoxicity, F_{LAC} values typically range between 40% and 60% of the reference. Sharper reductions are observed for fossil resource scarcity and water consumption (~30–40% of the benchmark), consistent with lower solvent and electricity requirements per functional unit as seen in the process trees for F_{LAC} . Hotspots for F_{LAC} are land use, marine eutrophication and ionizing radiation (≈ 60 –70%), mainly linked to the supply chains of vinyl/aromatic monomers (e.g., 4-aminostyrene, styrene, MMA) and the background electricity mix. In the benchmark, the process trees show more intensive solvent and electricity use, plus notable aqueous streams; as noted, excluding chitosan and EDC likely underestimates its true burden. Figure 7 corroborates the trend at endpoint level, with ~55–70% integrated reductions for F_{LAC} : ~45% (Human health), ~43% (Ecosystems) and ~31% (Resources), the strongest improvement being in Resources.

The comparison is made with full respect to the benchmark study, which provides an effective and stable immobilisation route; our work advances the state of the art by showing that an alternative support (F_{LAC}) can improve environmental viability for the same function. Overall, guided by the process trees and the dominant categories, the results support F_{LAC} 's environmental promise and outline an eco-design pathway: (i) greener support chemistry (optimize/replace 4-aminostyrene/styrene and MMA; safer alternatives to azide blocking; >90% solvent recovery), (ii) decarbonize the electricity background, and (iii) increase batch yield and effective re-use of the film to dilute impacts per functional unit. Given the attributional nature and bench scale with a very small

functional load, values should be read as comparative signals; nevertheless, the consistent reductions across midpoints and endpoints indicate that FLAC offers substantive environmental advantages and a clear roadmap towards pilot scale.

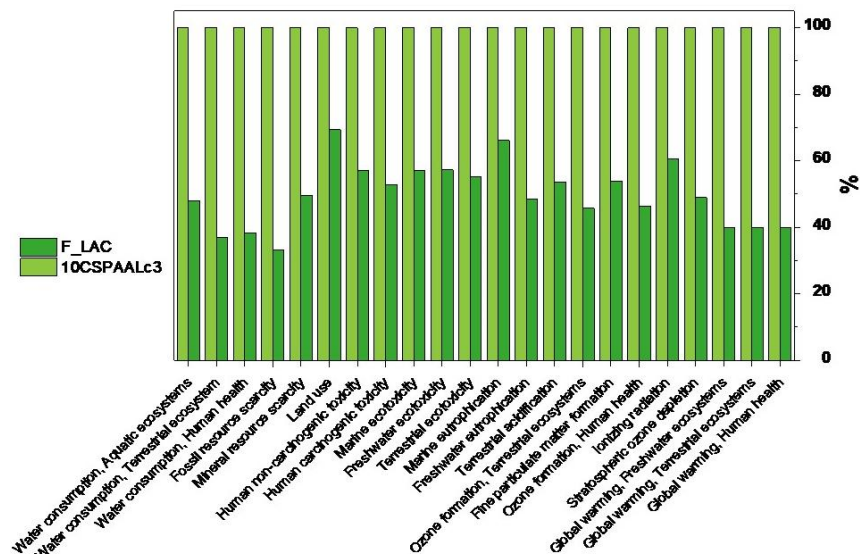


Figure 6. Comparative environmental impacts of the F_{LAC} and reference biocatalysts (10CSPAALc3) for the degradation of 50 µg of indigo carmine grouped, by impact categories, calculated using the ReCiPe 2016 Endpoint (H) method.

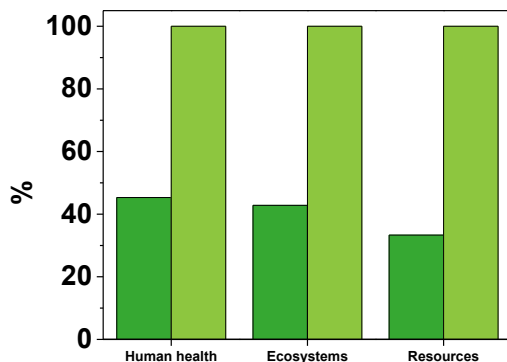


Figure 7. Comparative environmental impacts of the F_{LAC} and reference biocatalysts (10CSPAALc3) for the degradation of 50 µg of indigo carmine, by damage categories, calculated using the ReCiPe 2016 Endpoint (H) method.

3.8. TEA Study

3.8.1. Area-based performance metric (unit-level)

Figure 8a displays the area-normalised productivity as a function of cycle time for the three scenarios (3 discs, 3 mL; $\frac{V}{A_{tot}} = 35.37 \text{ L} \cdot \text{m}^{-2} \cdot \text{cycle}^{-1}$). The second-order polynomial fit (red dashed line) captures the expected curvature from $\Pi_{A,t} \propto t$ and is given by $y = 11.865 t^2 - 61.254 t + 88.74$. Within this framework, Scenario 3 ($t \approx 0.67 \text{ h}$) reaches $53.05 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$, lying in the region of maximal slope (high marginal gain per additional time reduction) and very close to the empirical upper envelope over the measured window. Longer cycles deliver lower productivities: Scenario 2 (1.50 h) $23.58 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$, and Scenario 1 (3.00 h) $11.79 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$, consistent with the non-linear trend. Quantitatively, our improvement efforts deliver an $\sim 350\%$ increase in area-normalised productivity relative to the baseline ($11.79 \rightarrow 53.05 \text{ L} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$; Scenario 1 $\rightarrow$ 3), attributable to optimising the surfactant-containing medium and the operational settings.

From a Techno-Economic Analysis (TEA) and sizing perspective, this representation confirms that shortening the cycle (e.g., via surfactants in the medium) is a more effective lever than increasing mediator dose: at 1 ppm Syr, Scenario 3 attains $\sim 4.5\times$ the baseline productivity and $\sim 2.25\times$ that at 2 ppm. The polynomial fit is empirical and, with three data points, reproduces the data exactly ($R^2 = 1$); therefore, extrapolation beyond 0.67–3.00 h should be treated with caution because the practical ceiling will be set by mass-transfer limitations and enzyme turnover, rather than by the polynomial intercept. However, the present laboratory results suggest the system is already operating close to its optimal productivity ceiling under the conditions studied. Taken together, the figure underscores that medium composition (presence of surfactants) is decisive for maximising area-normalised productivity and, consequently, for reducing the required area (associated CAPEX) and the operating time (time-dependent OPEX) per unit volume treated.

3.8.2. Cost of solution per gram of dye removed (mediator-free)

This was the first step to compute the second indicator (See Table 3; Unit cost build-up per disc by process stage): the per-disc unit cost was decomposed exclusively into raw materials and was allocated by process stage (polymer synthesis, diazotization, enzyme immobilization, azide blocking). The following assumptions were made: (i) only materials costs were counted (energy, labour, utilities, waste handling and mediator were excluded); (ii) batch-to-unit allocation was set by the reported yields (150 discs for polymer synthesis, 10 for diazotisation, 6 for immobilization, 10 for azide blocking); (iii) distilled water was not costed (negligible/OSBL); and (iv) catalogue unit prices were used in €/g (or €/mL). The resulting total per-disc cost was €0.019, with polymer synthesis as the dominant contributor (€0.012; 64.33%). Within that stage, aminostyrene (SNH_2) accounted for €0.010 per disc (57.16% of the total) and was therefore identified as the primary raw-materials driver. The remaining contributions were diazotization 15.52%, azide blocking 11.48%, and enzyme immobilization 8.67%.

For the TEA, this breakdown indicates the main levers: batch yield should be increased (to reduce allocation per unit), the costly monomer (SNH_2) should be optimized or substituted, and procurement at scale should be pursued—measures expected to give the largest marginal reductions in €/g, particularly under multi-reuse operation and/or higher treatment throughput.

Table 3. Unit cost build-up per disc by process stage

Stage	Cost per-disc (€)	Share of total (%)
Polymer synthesis	0.012153791	64.33
Diazotisation	0.002933000	15.52
Enzyme immobilisation	0.001637500	8.67
Azide blocking	0.002170000	11.48
Grand total per disc	0.018894291 ~ 0.019	100.00

Figure 8b displays, as a function of re-use number N , the per-cycle efficiency (left axis) and the film's specific cost in €/g (right axis), computed with a fixed raw-materials cost for three discs $C=0.056682873$ € and an IC mass per cycle of 3.0×10^{-5} . Over the first five uses, efficiency remains high (100–100–100–90–87 %), while the €/g decreases from 1889 to 396 (–79%, $\approx 4.8\times$ improvement). This behaviour follows directly from the denominator (cumulative treated mass) increasing with N while the numerator remains constant. The analysis identifies the service life of the support (re-use) as a primary lever in the TEA: there is a natural operating window of $N=3$ – $5N$ in which the largest marginal reduction in cost is achieved with minimal efficiency loss. The end-of-life (EOL) definition—e.g., setting a minimum acceptable efficiency of 80%—will determine the maximum N and hence the final €/g; with the observed profile, this threshold is not reached within five re-uses, and €/g continues to decline. Accordingly, extending service life and, in parallel, increasing the volume or concentration per cycle, emerge as critical strategies to enhance the techno-economic viability of the system.

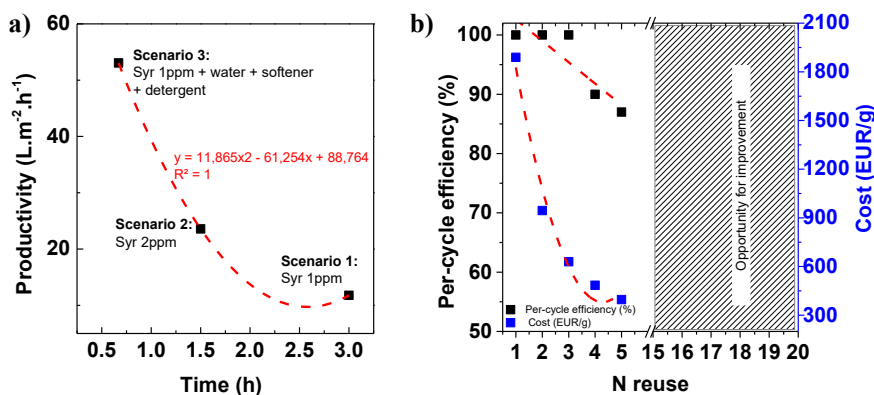


Figure 8. a) Area productivity vs time; polynomial fit and near-ceiling performance in a surfactant medium. b) Effect of re-use number on per-cycle efficiency (left) and film specific cost (€/g, right); the hatched area denotes the opportunity for improvement.

When energy and labour are included in film fabrication, the total cost allocated to three discs is €10.21268 (energy: 0.682 kWh $\rightarrow$ €0.136; labour: €10.02; raw materials: €0.05668). Using this numerator, the specific cost is €340,422.76 per

g (single use, 100%), €341,104.97 per g (99.8%), and €71,367.46 per g with five re-uses, see Table SI7 and SI8. Labour dominates the numerator, energy is secondary, and the raw-materials price has a marginal effect under these conditions. The absolute figures are large because the study sits at low TRL/bench scale, where (i) the mass treated per cycle is very small (30 µg), (ii) production is manual and batch-wise with high operator time, and (iii) fixed costs are allocated to few discs. The TEA therefore points to clear priorities: increase batch yield (more discs per polymerization), parallelise/automate wet-chemistry steps to reduce labour per unit, and extend service life (re-use); in parallel, increase volume or concentration per cycle and shorten cycle time in suitable media to grow the denominator and improve €/g. Overall, these results are not intended as definitive industrial costs but to expose the bottlenecks and chart the pathway so that, at pilot/demonstration scale, €/g falls substantially and the cost structure shifts away from labour towards inputs and equipment representative of scale-up.

4. Conclusions

This study presents a cost-effective and manageable immobilized laccase polymeric material (FLAC) for efficient dye degradation, with significant potential for industrial and environmental applications. Syringaldazine (Syr) was identified as the most efficient mediator, achieving high degradation rates even at minimal concentrations. The novelty of this work lies in the use of a film-shaped polymeric support that is robust, reusable, and easy to handle, combined with the exclusive application of natural mediators as a greener alternative to synthetic ones such as ABTS or HBT. Another important innovation is the brief exposure of the dye solution to FLAC for just 15 minutes, after which the degradation process continued autonomously even after removing the material. This short-exposure strategy, reported here for the first time to our knowledge, enhances efficiency and enables multiple reuse cycles. The system demonstrated excellent reusability, retaining over 90% of its activity after several cycles, and its performance was notably enhanced in the presence of detergents and softeners,

with complete dye degradation achieved in under an hour. These improvements under realistic washing conditions confirm the contribution of this work to bridging laboratory studies and real-world applications, offering a low-cost and sustainable solution for textile wastewater treatment. The unit-level TEA shows that shortening the cycle in surfactant media boosts area-normalized productivity by ~350%, and re-use ($N \approx 3-5$) lowers €/g by ~5×; at bench scale, €/g is dominated by labour and aminostyrene, prioritizing higher batch yield and automation. The comparative A-LCA indicates that F_{LAC} achieves ~55–70% reductions at the endpoint level (~40–60% at midpoints) versus the literature benchmark, with hotspots in land use, marine eutrophication and ionizing radiation linked to monomers and the electricity mix. Together, both assessments identify the improvement levers: shorten the cycle, maximise re-use and per-cycle volume/concentration, increase batch yield and automate, manage the mediator, green the support chemistry and recover solvents, and decarbonise energy, defining a pathway to pilot with substantial reductions in €/g and in the environmental footprint.

Data Availability

Open Data is available at <https://riubu.ubu.es/handle/10259/5684> under the name “UBU-Polymers Research Group 21032024”.

Competing interests

The authors declare no competing interests.

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Supplementary Material

Materials, instrumentation and methods; life cycle inventory; detailed methodology and equations for the techno-economic analysis (TEA)

The Supplementary Material is available free of charge at:

<https://ars.els-cdn.com/content/image/1-s2.0-S2590123025039325-mmc1.pdf>

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Multifunctional smart polymers and citizen science for a comprehensive approach to nitrate pollution: curative and preventive strategies

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Multifunctional smart polymers and citizen science for a comprehensive approach to nitrate pollution: curative and preventive strategies

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Abstract

This work presents the development and evaluation of a multifunctional smart polymer (FNO₃) for the extraction and detection of nitrates in drinking water. A total of 250 tap water samples from various localities were analysed, revealing nitrate concentrations that in some cases doubled the legal limit (up to 100 mg·L⁻¹). FNO₃, composed of 49.75 mol% NNZA monomer with high anion-exchange capacity, exhibited a maximum nitrate adsorption capacity (q_{max}) of 164 ± 5 mg·g⁻¹, which is 3.6 times greater than that of commercial resins. The polymer demonstrated significant swelling in water (~2014 ± 152%) and incorporated a sensing functionality via a fluorometric monomer, enabling visual detection when saturation occurs. Fluorescence response studies yielded a limit of detection (LOD) of 4.26 mg·L⁻¹ and a limit of quantification (LOQ) of 12.92 mg·L⁻¹, values that are below the regulatory thresholds established by European and Spanish legislation for nitrates in drinking water. The material was tested through multiple adsorption-regeneration cycles using domestic saline solutions, maintaining stable efficiency. Interference studies indicated that carbonates present in hard water partially reduce adsorption effectiveness. *Life Cycle Assessment (LCA)* identified the structural materials and functional monomers as

the main contributors to environmental impact, while reuse and polymer application offer environmental benefits due to nitrate recovery. Additionally, in vitro toxicological assays with *HepG2* cells confirmed the absence of cytotoxicity, supporting the polymer's viability for safe water treatment applications.

KEYWORDS: Nitrate removal; nitrate detection; ion exchange polymer; resin; smart polymers; water treatment; fluorescent sensor; toxicity assessment; life cycle analysis (LCA)

1. Introduction

Nitrate pollution, mainly caused by the overuse of fertilizers and poor management of organic waste, represents a growing threat to ecosystems, human health, and climate stability.¹ Nitrogen-rich compounds, particularly nitrates, infiltrate water bodies and soils through agricultural runoff, untreated sewage, and livestock waste. Once in the environment, these pollutants trigger a cascade of harmful effects.

In aquatic ecosystems, elevated nitrate levels lead to eutrophication, promoting algal blooms that deplete oxygen, kill fish, and reduce biodiversity. On land, nitrates disrupt nutrient cycles and plant communities. Human health is also at risk: high nitrate concentrations in drinking water are associated with methemoglobinemia (or "blue baby syndrome") in infants,² and potentially with an increased risk of certain cancers in adults.^{3,4} Furthermore, nitrate pollution contributes to the emission of nitrous oxide, a potent greenhouse gas, aggravating climate change.

Despite its severity, monitoring nitrate pollution remains a major challenge. Current monitoring systems are fragmented, producing low-resolution data and leaving many rural and agricultural areas without coverage. This lack of reliable data hinders effective policymaking and the implementation of targeted interventions. Global institutions such as the UN Water Conference and UNEP have emphasized the urgent need for improved water quality data to track progress toward Sustainable Development Goal 6.3.2. Although tools like the

SDG Water Quality Hub attempt to consolidate existing information (“Water and Sanitation - United Nations Sustainable Development,”), major gaps in availability and comparability persist.

To help address these gaps, this work explores a citizen science approach from a preventive perspective. This initiative has had significant public impact, with dozens of appearances in national media, reflecting growing social concern and awareness about nitrate pollution in rural environments.^{6,7}

However, monitoring alone is not enough—access to safe drinking water remains a fundamental right, as recognized in Spanish law (Law 7/1985, Art. 26).⁸ Yet, our study found evidence that this right is not being fulfilled in several municipalities, where nitrate levels exceed safe limits.

Current curative technologies, such as reverse osmosis and ion-exchange resins, are widely used to remove nitrates from drinking water, especially in domestic systems. However, these methods are non-selective, removing beneficial minerals alongside nitrates, often requiring post-treatment remineralization. More critically, they are wasteful and unsustainable: reverse osmosis can waste up to 10 litres of water for every litre purified, and both systems generate secondary waste streams and demand high operational costs, making them unsuitable for rural or small-scale applications.

To overcome these limitations, this study presents a novel solution: the use of reusable smart polymer materials for decentralized nitrate removal. Our material (FNO_3) consists in functionalized polymeric films capable of removing nitrates from water with high affinity under typical conditions, showing a preferential interaction with nitrate ions over other common anions such as bicarbonate anions. Once saturated, the films could be returned through a subscription service, where the captured nitrates would be recovered and reused as fertilizer in local agricultural businesses, embedding circularity into the process. Moreover, these smart materials are multifunctional; in addition to removing nitrates, they emit a fluorescent signal upon saturation, indicating the need for regeneration and thereby facilitating their use by non-specialized

personnel. Thus, the smart material proposed in this work meets both classifications established by García et al.,⁹ namely, it functions as both a reactive polymer and a sensory polymer.

In recent years, a specific class of polymer-based smart materials—unsupported thin films, meaning films that are not applied onto inert substrates but rather constitute the final functional material themselves—have emerged as promising tools in a wide range of applications,¹⁰ including biomedicine,^{11,12} food safety,^{13,14} and, as explored in this work, environmental monitoring.^{15,16} These materials are synthesized via random radical polymerization directly within the final mold, differing from conventional thin films often fabricated by casting or spin-coating techniques. A key feature of this approach is that no external additives are used during synthesis: all reagents are monomers, crosslinkers, or initiators that become covalently integrated into the main polymer backbone. This additive-free composition minimizes the risk of chemical leaching, which is particularly relevant for water-contact or food-contact applications. Furthermore, the random arrangement of polymer chains has been shown to enhance performance, often surpassing that of traditional small-molecule colorimetric or fluorometric probes.

To the best of our knowledge, this material represents a significant innovation, as it operates without the need for electricity,^{17,18} specialized equipment,^{17,19} or electrodes^{20,21}—unlike other methods and materials reported in the scientific literature. Another key distinguishing feature is that the fluorescence-switching reaction is reversible, in contrast to many commercial nitrate-detection test strips, which typically rely on irreversible diazonium coupling reactions.²² Furthermore, the material itself integrates visual detection capability with effective nitrate removal from water, which, to the best of our knowledge, makes it a unique solution within its category.

This approach merges advanced materials science with citizen-centered design, creating a low-cost, scalable, and sustainable alternative to traditional water treatment systems. By integrating community monitoring with

decentralized, circular purification technology, our material offers a holistic response to the challenges of nitrate pollution—empowering local action while supporting environmental and economic resilience.

Based on the context described above, the specific objectives of this work are: (i) to develop a multifunctional polymeric thin-film material (FNO_3) capable of selectively removing nitrates from water while providing a visual fluorescence signal for saturation detection; (ii) to evaluate the material's performance in terms of nitrate extraction efficiency, selectivity, reusability, and limits of detection and quantification under laboratory and realistic conditions; (iii) to implement a citizen science approach for monitoring nitrate pollution, assessing public engagement, and promoting awareness and preventive action in rural communities; (iv) to assess the sustainability and potential environmental impact of the proposed material through preliminary *Life Cycle Assessment (LCA)* and toxicological evaluation, ensuring the proposed solution is environmentally safe and scalable.

2. Experimental

2.1. Materials

All materials and solvents were commercially available and used as received unless otherwise indicated. The following materials and solvents were used: 1-vinyl-2-pyrrolidone (VP) (Acros Organic, 99%), methylmethacrylate (MMA) (Merck, 99%), 5-bromopentene (TCI, 95%), 6-methoxyquinoline (Thermo Scientific, 98%), methanol (VWR, 99.8%), diethyl ether (VWR, 99.7%), (vinylbenzyl)trimethylammonium chloride (NNZA) (Sigma-Aldrich, 99%), hydrochloric acid (VWR, 37%), chloroform (VWR, 99%), ethylene glycol dimethacrylate (E) (Aldrich, 98%), 2,2-Dimethoxy-2-phenyl-acetophenone (Aldrich, 99%), potassium chloride (Sigma-Aldrich 99.5%), potassium nitrate (VWR, 99.7%), sodium carbonate (Sigma-Aldrich, 99.9%), nitrate exchange commercial resin (Dupla, Duresin N 80563).

2.2. Instrumentation and general methods

The water-swelling percentage (WSP) of the films, defined as the percentage of water absorbed by the films upon immersion in pure water at 20 °C until equilibrium, was determined using the dry film weight (ω_d) and its water-swelled weight (ω_s) with the equation 1 (eq. 1):

$$\text{WSP} = 100 \times [(\omega_s - \omega_d) / \omega_d] \text{ (eq. 1)}$$

The thermal behaviour of the polymers was analyzed using thermogravimetric analysis (TGA) performed on a Q50 TGA analyser (TA Instruments, New Castle, DE, USA) with 10–15 mg of sample under synthetic air and nitrogen atmospheres at a heating rate of 10 °C·min⁻¹.

Fourier-transform infrared (FTIR) spectra were recorded using an FT/IR-4200 infrared spectrometer (Jasco, Tokyo, Japan) equipped with an ATR-PRO410-S single-reflection accessory.

Differential scanning calorimetry (DSC) was carried out using a TA Instruments Q200 DSC analyser with a heating rate of 20 °C·min⁻¹ under a nitrogen atmosphere and 10-15 mg of sample.

¹H and ¹³C{¹H} NMR spectra (Advance III HD spectrometer, Bruker Corporation, Billerica, Massachusetts, USA) were recorded at 300 MHz for ¹H and 75 MHz for ¹³C using deuterated dimethylsulfoxide (DMSO-*d*₆) at 25 °C as the solvent.

The limit of detection (*LOD*) and the limit of quantification (*LOQ*) were estimated using the equations 2 and 3:

$$\text{LOD} = 3.3 \times SD / s \text{ (eq. 2)}$$

$$\text{LOQ} = 10 \times SD / s \text{ (eq. 3)}$$

where *SD* is the standard deviation of a blank sample and *s* is the slope of the calibration curve in a region of low nitrate content.

2.3. Tap water sampling through citizen science

This study combined a large-scale citizen science initiative with a year-long intensive monitoring campaign in two selected towns. The citizen science component involved the collection of 557 water samples from multiple locations across the province of Burgos, Spain, covering an area of 14,022 km², and representing diverse water sources and towns. These sources included streams, springs, and other natural bodies; however, the primary focus was on tap water samples, as they are most relevant for evaluating nitrate exposure in domestic contexts. Tap water corresponds to treated water supplies—most commonly disinfected with sodium hypochlorite—whereas natural sources (springs, streams, rivers) are untreated. This focus also responds to the current Spanish regulation (Royal Decree No. 3/2023), which only applies to drinking water; natural sources such as rivers, springs, or streams fall outside the scope of the law, as they are not directly considered for human consumption. Nevertheless, given the high level of public concern, we also analyzed non-regulated natural sources (springs, streams, rivers), and these additional results—showing even higher incidences of non-compliance—are provided in the Supplementary Material (SM-Section S1).

Of the total, 250 valid tap water samples were collected from different municipalities. It is worth noting that many of the analyzed samples were duplicates originating from the same locality, and numerous others had to be discarded due to non-compliance with the sampling protocol established by our research group.

In parallel, a year-long monitoring program was conducted in the towns of Briviesca and Avellanosa del Páramo. Samples were collected approximately every four weeks throughout 2024 to capture temporal variability in tap water quality. While full experimental details are provided in SM-Section S1, a summary of the sampling approach is presented below.

For both components, the sampling protocol was simple and accessible. Participants were instructed to use a sterile sampling container or, alternatively,

a standard 500 mL plastic bottle originally intended for mineral water. If using a plastic bottle, it had to be emptied and not drunk from directly. The container was to be rinsed three times with tap water from the participant's home to minimize contamination and then filled completely. The only identifying information provided with each sample was the name of the town where it had been collected.

Once collected, the samples were delivered to our laboratory facilities, where they were analyzed as described in the following sections. The results of the analyses were subsequently communicated to the corresponding local municipalities via their official institutional email addresses.

2.4. Quantification of nitrate in water samples

The determination of nitrate concentration in real water samples was carried out using UV spectrophotometry at 220 nm (Hitachi U-3900 UV/Vis spectrophotometer (Hitachi High-Tech, Japan), following the general guidelines of the Standard Methods for the Examination of Water and Wastewater (Method 4500-NO₃⁻ B), with specific adaptations detailed in SM-Section S2.

2.5. Design of the multifunctional smart polymer

The citizen science project not only highlighted the scope of the nitrate contamination problem but also provided insight into current solutions and the specific demands of the public regarding feasible technologies. From this, we identified key limitations in the two most commonly used household systems: reverse osmosis and ion exchange units.

While reverse osmosis systems are compact and easily installable, they often produce wastewater and can be expensive to maintain. Ion exchange resins, on the other hand, are expensive, but robust and durable, and can be regenerated with relatively simple procedures. Our approach therefore aimed to combine the advantages of both strategies.

We developed a smart material (FNO₃) inspired by the chemistry of ion exchange resins, incorporating a high proportion of NNZA-based moieties (49.75 mol%) into its structure to ensure high anion-exchange capacity (Figure 1). To

render the polymer structurally manageable and compatible with everyday use, we selected a formulation including hydrophilic N-vinylpyrrolidone (VP, 25 mol%) and hydrophobic methyl methacrylate (MMA, 25 mol%).

This balance, combined with the use of an appropriate cross-linker, resulted in a robust and easy-to-handle material, despite its high water swelling %, which reaches approximately $2014 \pm 152\%$, relatively high for this type of materials designed for environmental control applications¹⁶ (more information about the synthesis and characterization of the polymer FNO₃ can be found in SM-Section S3).

To facilitate public engagement and use, FNO₃ was designed as a simple, immersible film that passively extracts nitrate from water. A key concern, however, was user feedback—how can users know when the film needs to be replaced or regenerated? To address this, we incorporated a small fraction (0.25 mol%) of the SA-R1025 monomer, a fluorometric sensor responsive to chloride anions²³ (more information about the synthesis of the monomer in SM-Section S4). The underlying sensing mechanism relies on dynamic quenching: when the polymer is in its fresh state, the NNZA sites are paired with chloride counterions that quench the fluorescence of the quinolinium core in SA-R1025. As nitrate ions replace chloride ions during the exchange process, quenching decreases and fluorescence re-emerges. This visual signal provides a straightforward and user-friendly indicator that the film has reached saturation and should be replaced or regenerated via immersion in a KCl solution.

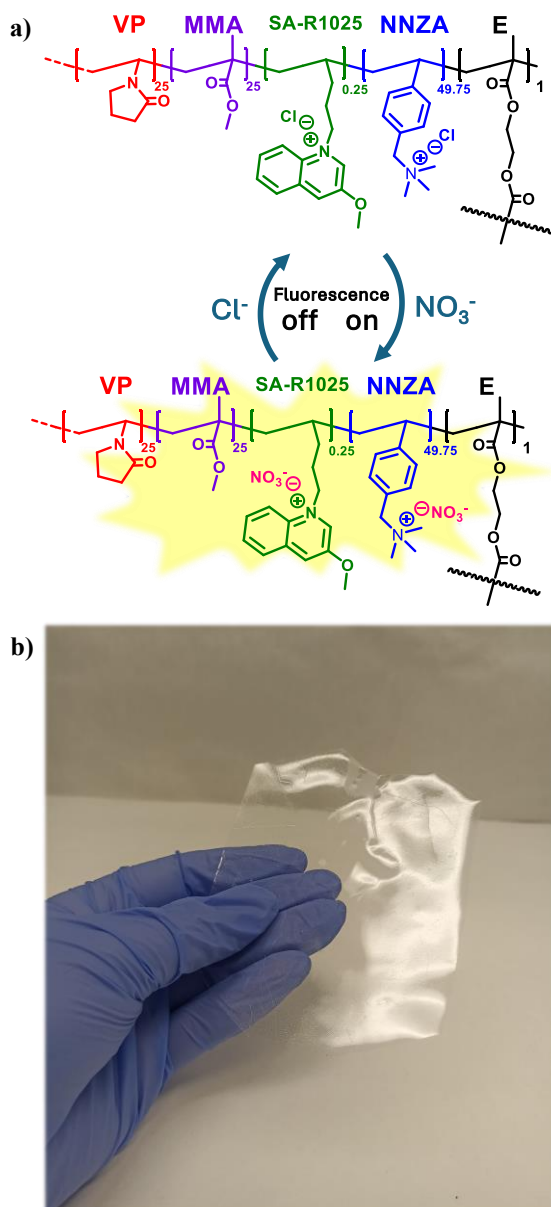


Figure 1. a) Chemical structure of the multifunctional smart copolymer designed for nitrate removal, composed of 49.75 mol% NNZA units (anion-exchange sites), 25 mol% VP (hydrophilic component), 25 mol% MMA (providing hydrophobic rigidity), and 0.25 mol% SA-R1025 (fluorescent sensor unit). b) The photograph shows the final material (FNO₃) in its film-like format.

2.6. Sorption study

The adsorption kinetics were monitored using absorbance measurements. Specifically, the decrease in nitrate concentration over time was tracked upon immersion of FNO₃ in the solution. To determine nitrate concentration at each time point, a calibration curve was established based on absorbance readings.

11 potassium nitrate (KNO₃) solutions were prepared, with nitrate anion concentrations ranging from 5 to 100 ppm using stock solutions prepared in section 2.4. In this case, a wavelength of 222 nm was selected, ensuring that absorbance saturation did not occur at any concentration. All measurements were performed using a quartz cuvette filled with 3.5 mL of each solution. A 10 mm diameter disc (1.6 mg), obtained by punching directly from the polymer film, was introduced into the cuvette, and nitrate removal was monitored at 25 °C over a period of 500 minutes. Each concentration was tested in triplicate.

Using the obtained values of q_e (milligrams of adsorbate adsorbed per gram of adsorbent at equilibrium) and C_e (concentration of adsorbate remaining in solution at equilibrium), from the different adsorption kinetics, the adsorption isotherm was constructed by averaging these values across the three replicates performed for each concentration.

2.7. Fluorescence and interference study

To investigate the sensory behavior, the fluorescence enhancement of FNO₃ was monitored over time at different nitrate concentrations (50–500 ppm, in MQ water) using a Hitachi F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The procedure followed a method analogous to that used in the kinetic study, maintaining the same solution volume, amount of FNO₃, and experiment time.

Interference study: drinking water contains various dissolved salts, predominantly calcium and magnesium salts, which contribute to different degrees of water hardness. Hardness is commonly expressed in mg·L⁻¹ of sodium carbonate. Accordingly, five different carbonate concentrations (50, 100, 200, 300 and 500 ppm) were prepared in MQ water to study the removal of

nitrates —initially at 95 ppm— by UV-Vis spectrometry before and after the treatment with FNO_3 . The experiments were conducted using a solution volume of 3.5 mL and 1.6 mg of FNO_3 .

Fluorescence images of FNO_3 shown throughout the article were taken with an iPhone 16 under ultraviolet light irradiation at 365 nm.

2.8. Reusability study

In this study, a procedure analogous to that used in adsorption isotherm analysis (section 2.6) was followed to evaluate the reusability of FNO_3 , designed for nitrate extraction from drinking water (500 min, 25 °C). A fixed amount of 1.6 mg of FNO_3 was employed with a final volume of 3.5 mL of polluted water.

The water originated from Avellanosa del Páramo (Spain), and its salt composition was determined through external analysis conducted by the company *DXD Soluciones Químicas S.L.* Absorbance measurements of the water were taken before and after the addition of FNO_3 to quantify the extraction of nitrates and other ions present. To prevent measurement interference from carbonates, hydrochloric acid (HCl) was added after treatment with FNO_3 , and prior to UV-Vis analysis.

The adsorption process was carried out for a minimum of 500 minutes to ensure equilibrium. Subsequently, FNO_3 was regenerated by immersing it in a 500 mg·L⁻¹ potassium chloride solution for 120 minutes. After regeneration, FNO_3 was reused for a new purification cycle, repeating the process for a total of seven cycles.

2.9. Life Cycle Assessment study

This study employs a *LCA* to evaluate the environmental performance of a nitrate removal film for drinking water, considering both production and operational use phases. The functional unit is defined as 1 m³ of treated water, enabling comparison with equivalent technologies.

The assessment was conducted following the Environmental Footprint 3.1 (EF 3.1) methodology, in accordance with ISO 14040 and ISO 14044 standards and the European Commission's recommendations (2013/179/EU). This method integrates classification, characterization, normalization, and weighting steps to derive a single environmental impact score.

The analysis comprises two stages: **Production**: synthesis of functional monomers (NNZA, SA-1025); use of polymers and additives (EGDMA, MMA, VP, DMPA); structural materials (glass, stainless steel, polyethylene); energy consumption; transportation; emissions; industrial waste; and water use. Final disposal is excluded. **Use**: transportation to the point of consumption; water consumption for regeneration; valorization of nitrate-rich effluent as fertilizer (modelled as an avoided product); and minor waste generation (plastics, syringes). End-of-life management will be evaluated in a subsequent study.

Inventory data were obtained from experiments, literature sources, and the Ecoinvent v3.8 database using a "cut-off" allocation approach. For monomers unavailable in the database, specific processes were modelled according to synthesis routes, estimating emissions and by-products by analogy.

Impact assessment was performed using SimaPro v10.2.0.1, considering categories such as climate change, human toxicity, aquatic ecotoxicity, acidification, and use of mineral and fossil resources.

2.10. Toxicology study

2.10.1. Cell line and culture conditions

The human hepatocellular carcinoma epithelial-like cell line (HepG2) was cultured in Eagle's Minimum Essential Medium (EMEM), which was prepared as follows: commercial Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 1% non-essential amino acids (NEAA), and 1% sodium pyruvate (Gibco). The cell culture was kept in a thermostatic incubator under optimal growth conditions (humidified atmosphere containing 5% CO₂ and 37 °C).

2.10.2. Cell viability toxicology assay

- Water samples preparation

The developed material FNO_3 was used to treat a real water sample unsuitable for human consumption, collected from Avellanosa del Páramo (Spain). The water's toxicological profile was assessed both before and after exposure to the film. The potential of the water samples—WA (untreated Avellanosa water) and WA+C (Avellanosa water exposed to the film)—to affect cell viability was evaluated using HepG2 cells exposed to the maximum concentrations of each sample, which correspond to 90% of the final volume.

Prior to testing, water samples were filtered using $0.22\ \mu\text{m}$ PES filters. Subsequently, the culture medium, prepared at a 10X concentration, was diluted directly into the water samples to conduct the various assays.

- MTT Assay

HepG2 cells were seeded in 96-well plates at 1×10^4 cells per well and incubated for 24 hours. Cells were washed with Phosphate-Buffered Saline (PBS) and then treated with 200 μL of the maximum water concentration diluted in culture medium supplemented as explained above. As controls, cells incubated with culture medium alone (NC = live cells control) and cells treated with water only (PC = dead cells control) were used. 24 h after exposure, the culture medium containing FNO_3 was withdrawn, and cells were washed with PBS, and then 100 μL of a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-(2,5-diphenyl)tetrazolium bromide) (Sigma) at $500\ \text{mg}\cdot\text{L}^{-1}$ using no-supplemented culture media were added to each well, and incubated for 3 h, protecting cells from the light. After this time, the solution medium was discarded, and 100 μL of dimethyl sulfoxide (DMSO) were added per well to solubilize the formazan crystals by gentle shaking for 15 min at 500 rpm at room temperature, keeping the plate protected from light. Finally, the absorbance was measured using a BioTek Synergy HT microplate reader (BioTek Instruments, Winooski, VT, USA) at OD 590 nm. Data represent the mean of three biological replicates, each with four technical replicates.²⁴

- Statistical analysis

The background absorbance was subtracted, and the values were normalised to the mean of the negative control, which is set to 100% viability. Statistical analysis data are presented as means $\pm$ standard deviation (*SD*). The Two-way analysis of variance (ANOVA) was used for Dunnett's multiple comparison test to compare every mean with the negative control sample, which is used as the internal water control sample nitrate-free. Statistical tests were carried out using Prism 8.0 (GraphPad Prism, GraphPad Software, Inc.). Differences were considered significant at $P < 0,05$. **** $P < 0,0001$.

3. Results and discussion

3.1. Results of nitrate monitoring in tap water through citizen science

Among the 250 valid samples analyzed (Figure 2a), 22% exceeded the maximum allowable nitrate concentration in drinking water, as established by Spanish and European regulations ($50 \text{ mg}\cdot\text{L}^{-1}$), which, under these standards, is classified as a "very severe" condition.^{25,26} Additionally, 30% of the samples presented concentrations above $30 \text{ mg}\cdot\text{L}^{-1}$, a threshold considered "severe" that, according to Royal Decree No. 3/2023,²⁵ may trigger a correction factor if the situation persists or worsens over time. Particularly concerning is the fact that many of the affected municipalities were unaware of the problem: neither the residents nor, apparently, the local authorities had prior knowledge of the water quality issues. This lack of information and awareness on the part of the administration highlights the urgent need to implement both corrective and preventive measures.

Furthermore, various scientific studies have already pointed out that the legal limit may be too lenient. Evidence suggests that concentrations above $10 \text{ mg}\cdot\text{L}^{-1}$ may pose health risks, particularly in relation to colorectal and prostate cancers.^{3,4} Based on this more conservative threshold, 48% of the monitored towns would be considered to have unsafe nitrate levels.

The results were sent via email to the respective municipal governments. In many cases, officials expressed surprise, as this was the first time they had

been alerted to the issue. Contrary to the assumption that this is a problem limited to small, depopulated rural villages—where limited municipal resources might explain the lack of oversight—this is not always the case. For instance, a sample provided by a citizen from Aranda de Duero, the third most populous city in the province of Burgos, revealed a nitrate concentration of $58 \text{ mg} \cdot \text{L}^{-1}$, exceeding the legal limit. This result was rapidly communicated to the local authorities and confirmed with a second analysis, highlighting that the currently available platforms for checking nitrate concentrations in water need improvement, and that the communication of these results to citizens should also be made more effective.

3.2. One-year monitoring of nitrate concentrations

A common argument presented by certain administrative bodies is that nitrate levels in drinking water are subject to significant variability throughout the year. This variability is often attributed to two primary factors: (1) the application of fertilizers during sowing periods in the early months of the year, and (2) rainfall events that facilitate the leaching of nitrates into groundwater sources, potentially impacting water catchments.

In our study, we conducted a year-long monitoring of two locations within the province of Burgos, with samples collected by two of our researchers residing in these municipalities. The findings revealed two distinct patterns (Figure 2b): (i) Avellanosa del Páramo: nitrate concentrations remained consistently high throughout the year, averaging approximately $100 \text{ mg} \cdot \text{L}^{-1}$ —twice the legal limit. This locality is situated on a plateau characterized by elevated, flat terrain with calcareous soils and sparse arboreal vegetation. The land use is predominantly agricultural, focusing on dryland farming with minimal industrial activity. The combination of intensive fertilizer use, limited groundwater renewal, and the absence of surface water bodies for dilution contributes to the area functioning as a persistent nitrate reservoir, indicating a chronic contamination issue that is challenging to remediate in the short term. (ii) Briviesca: in contrast, this locality exhibited seasonal variations in nitrate levels, with concentrations decreasing

between April and September (post-rainy season) and increasing from October to March. Briviesca is located in the valley of the Oca River, flanked by the San José and Los Pinos mountains, featuring a more varied topography with both mountainous and flat regions. The Oca River provides a continuous source of surface water, enhancing groundwater renewal and facilitating nitrate dilution. Additionally, the area has a higher population density and includes an industrial park, factors that also influence nitrate levels, particularly during periods of reduced precipitation.

Results indicate that nitrate concentrations in drinking water are not solely dependent on seasonal factors but are significantly influenced by the hydrogeological characteristics and aquifer dynamics of each municipality.

3.4. Sorption study. evaluation of nitrate removal performance

The adsorption process in solution consists of several sequential transport stages. First, the solute moves from the bulk solution toward a thin boundary layer surrounding FNO_3 , a step known as external transport. Next, the solute diffuses through this boundary layer to reach FNO_3 's surface, a process referred to as external mass transfer. Subsequently, the solute diffuses within the FNO_3 particles, known as intraparticle diffusion. Finally, the solute is adsorbed onto the active sites of FNO_3 . To analyse the adsorption kinetics in this study, two primary mathematical approaches were employed: the *Surface Reaction Model (SRM)* and the *Mass Transfer Reaction Model (MTM)*. The results of this preliminary analysis (more information in SM-Section S5), showed that the model that best fit the data was dual resistance (intraparticle and film diffusion). The results are showed in Figure 3a.

The experimental values of q_e and C_e obtained (Figure 3b) were fitted to the *Langmuir* adsorption isotherm, as described by equation (4).

$$q_e = \frac{q_{\max} K_L C_e}{1 + K_L C_e} \quad (\text{eq. 4})$$

Langmuir was the first to propose a kinetic theory of adsorption on a flat surface ²⁷. This model describes the adsorption of a monolayer on a

homogeneous surface, with a fixed number of sites where adsorption of a single molecule occurs. In this equation, q_{max} ($\text{mg} \cdot \text{g}^{-1}$) represents the maximum amount of adsorbate per unit weight of adsorbent required to form a complete monolayer on the surface, and K_L ($\text{L} \cdot \text{mg}^{-1}$) is the affinity constant of the adsorbate for the adsorbent binding sites. These two constants enable the calculation of the thermodynamic equilibrium constant, K_c , as the product of both, according to equation (5):

$$K_c = K_L q_{max} \quad (\text{eq. 5})$$

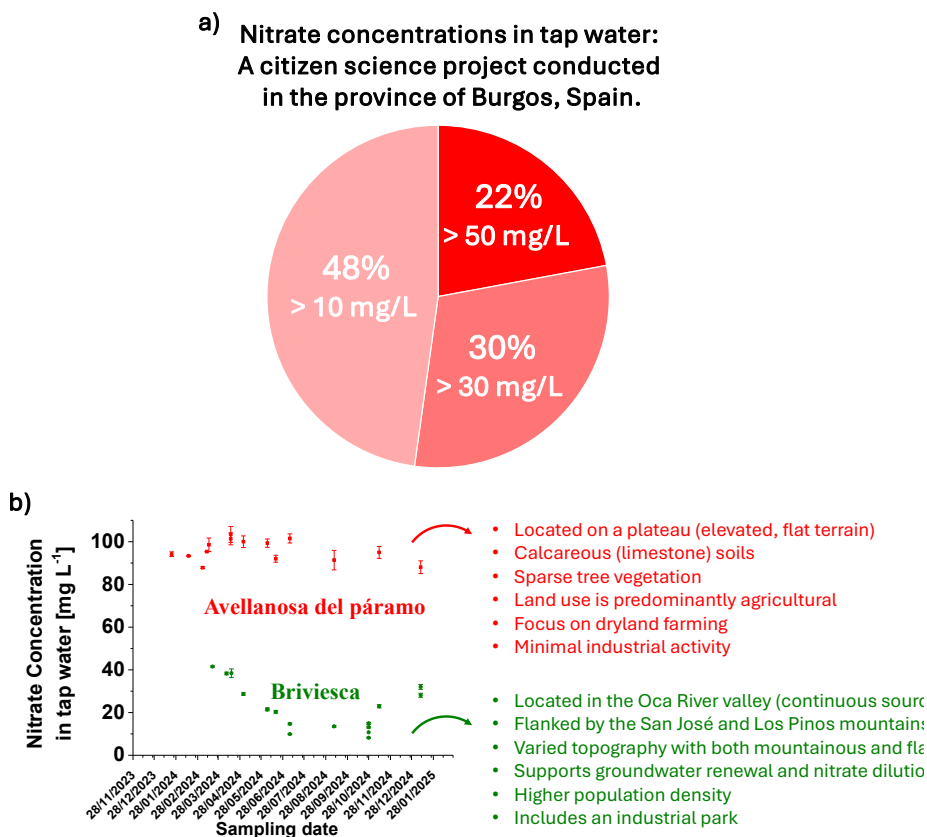


Figure 2. a) Graphical summary of the citizen science project conducted in the province of Burgos, Spain. b) Annual variation of nitrate concentrations in drinking water from Avellanosa del Páramo and Briviesca (Burgos, Spain).

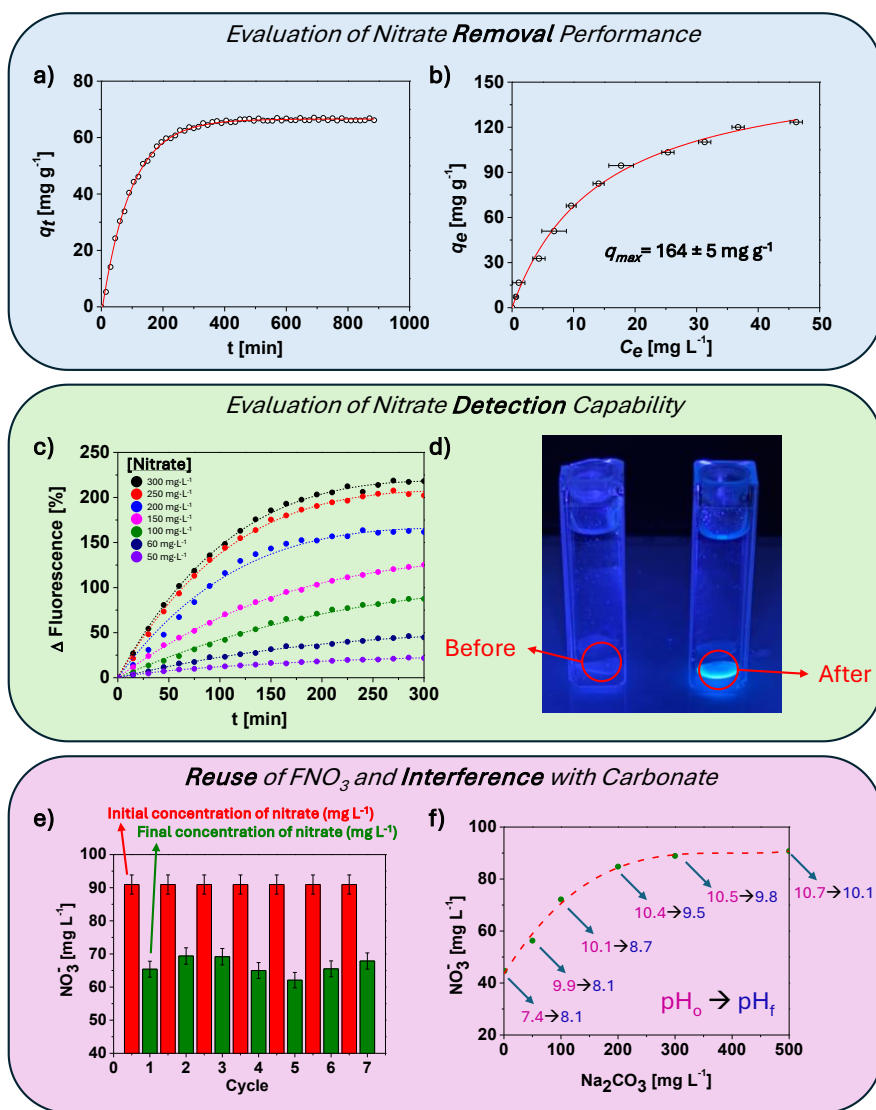


Figure 3. a) Crank's dual-resistance model applied to adsorption kinetics at 40 ppm and 25 °C. b) Langmuir adsorption isotherm at 25 °C. c) Time-dependent increase in fluorescence intensity after exposure to nitrate solutions of varying concentrations. d) Visual change in fluorescence of FNO₃ before and after exposure to a 100 mg·L⁻¹ nitrate solution. e) Reusability performance of FNO₃ over seven adsorption–regeneration cycles using real tap water. f) Interference study showing the effect of increasing carbonate concentrations on nitrate removal efficiency. The y-axis represents the nitrate concentration after treatment with FNO₃, starting from an initial nitrate concentration of 100 mg·L⁻¹. Each data point also displays the initial and final pH of the solution before and after treatment with FNO₃, respectively.

From equations (4) and (5), the following parameters were obtained: $K_L = 0.070 \pm 0.006 \text{ L} \cdot \text{mg}^{-1}$, $q_{\max} = 164 \pm 5 \text{ mg} \cdot \text{g}^{-1}$, $R^2 = 0.9952$, $K_C = 710 \pm 80$. It is worth noting that we calculated the $q_{\max}$ for a nitrate exchange commercial resin (Dupla, Duresin N 80563), obtaining a value of approximately $45 \text{ mg} \cdot \text{g}^{-1}$, therefore, our material exhibits a nitrate adsorption capacity that is 3.6 times higher than that of the commercial counterpart (“Dupla,”).

Using these values, it is possible to calculate the amount of adsorbent required to remove a specific quantity of adsorbate from a solution containing only nitrates, as shown in Table 1. This table is of high relevance, as it provides end users with guidance on the required amount of FNO_3 to use, based on the initial nitrate concentration in their local water supply and the desired final nitrate concentration after treatment with FNO_3 .

Table 1. Estimated amount of FNO_3 (g) required to treat 1 L of water with varying initial and final nitrate concentrations, based on Langmuir model parameters. The vertical column in bold indicates the initial nitrate concentration ($\text{mg} \cdot \text{L}^{-1}$) before using the FNO_3 film, and the horizontal row in bold indicates the final nitrate concentration ($\text{mg} \cdot \text{L}^{-1}$) after treatment.

		Desired nitrate concentration ($\text{mg} \cdot \text{L}^{-1}$) after treatment with FNO_3				
		50	40	30	20	10
Nitrate concentration ($\text{mg} \cdot \text{L}^{-1}$) before treatment with FNO_3	150	0.80 $\pm$ 0.10	0.90 $\pm$ 0.20	1.10 $\pm$ 0.20	1.40 $\pm$ 0.20	2.10 $\pm$ 0.30
	140	0.70 $\pm$ 0.10	0.80 $\pm$ 0.10	1.00 $\pm$ 0.20	1.30 $\pm$ 0.20	1.90 $\pm$ 0.30
	130	0.60 $\pm$ 0.10	0.70 $\pm$ 0.10	0.90 $\pm$ 0.20	1.20 $\pm$ 0.20	1.80 $\pm$ 0.30
	120	0.55 $\pm$ 0.10	0.70 $\pm$ 0.10	0.80 $\pm$ 0.10	1.00 $\pm$ 0.20	1.60 $\pm$ 0.20
	110	0.47 $\pm$ 0.09	0.60 $\pm$ 0.10	0.70 $\pm$ 0.10	0.90 $\pm$ 0.20	1.50 $\pm$ 0.20
	100	0.39 $\pm$ 0.07	0.50 $\pm$ 0.09	0.60 $\pm$ 0.10	0.80 $\pm$ 0.10	1.30 $\pm$ 0.20
	90	0.31 $\pm$ 0.06	0.41 $\pm$ 0.07	0.54 $\pm$ 0.09	0.70 $\pm$ 0.10	1.20 $\pm$ 0.20
	80	0.24 $\pm$ 0.04	0.33 $\pm$ 0.06	0.45 $\pm$ 0.08	0.63 $\pm$ 0.10	1.00 $\pm$ 0.20
	70	0.16 $\pm$ 0.03	0.25 $\pm$ 0.04	0.36 $\pm$ 0.06	0.52 $\pm$ 0.09	0.90 $\pm$ 0.10
	60	0.08 $\pm$ 0.01	0.17 $\pm$ 0.03	0.27 $\pm$ 0.05	0.42 $\pm$ 0.07	0.74 $\pm$ 0.11
	50	- - -	0.08 $\pm$ 0.01	0.18 $\pm$ 0.03	0.31 $\pm$ 0.05	0.59 $\pm$ 0.09

3.5. Fluorescence study. Evaluation of nitrate detection capability

In addition to removing nitrates from the medium, FNO_3 also undergoes a fluorescence turn-on (OFF–ON) process as the concentration of adsorbed

species increases. This phenomenon is primarily attributed to the presence of chloride ions within FNO_3 , which quench the fluorescence of the coumarin motifs. Upon displacement of the chlorides by nitrates, the fluorescence is restored. This behaviour not only enables the detection of nitrates in the medium but also allows their quantification.

Figure 3c illustrates the progressive increase in the fluorescence intensity of the material over time following its immersion in solutions with varying nitrate concentrations, ranging from 50 ppm to 300 ppm. A direct correlation is observed between the nitrate concentration in the solution and the emitted fluorescence intensity, confirming the ability of FNO_3 to respond sensitively and rapidly to changes in the concentration of this anion.

From a more qualitative perspective, Figure 3d displays images of the material before and after immersion in a nitrate solution at a concentration of $100 \text{ mg}\cdot\text{L}^{-1}$. These images demonstrate that the material enables straightforward and clear visual detection, facilitating its use by non-specialized users. This feature is particularly relevant for citizen science applications, where accessibility and ease of use are critical for participatory water quality monitoring.

Although the calculated limits of detection (LOD , $4.26 \text{ mg}\cdot\text{L}^{-1}$), and quantification (LOQ , $12.92 \text{ mg}\cdot\text{L}^{-1}$) are not exceptionally low—particularly when compared to commercial systems such as those developed by Hanna Instruments, which can achieve detection levels as low as $0.01 \text{ mg}\cdot\text{L}^{-1}$ of $\text{NO}_3\text{-N}$ —they are nonetheless adequate for the intended application.²⁹ This is because they remain more than an order of magnitude below the maximum concentration limits established by both European and Spanish water quality regulations.^{25,26} It is important to note that many of the most sensitive commercial systems rely on indirect detection methods involving the formation of azo dyes and absorbance measurements within the 340–800 nm range, which require derivatization steps. In contrast, our method allows direct nitrate detection based on fluorescence without the need for chemical derivatization, with the added advantage that the material cost is relatively low, approximately €1.7 per gram. For nitrate

concentrations around several milligrams per litre, the response time of the material ranges between 50 and 100 minutes, depending on the presence of potential interfering species. Furthermore, unlike commercial solutions that necessitate the purchase of specialized instrumentation and consumable reagents, our system operates without the need for either equipment or reagents, offering a simpler, more cost-effective, and user-friendly alternative for decentralized and participatory water monitoring.

3.6. Reusability of FNO_3 , carbonate interference, and pH influence

3.6.1. Reusability of FNO_3

One of the key requirements for FNO_3 is its reusability, ideally allowing the regeneration process to be performed at home using readily available household reagents, such as common table salt. In our case, we employed tap water from Avellanosa del Páramo (Spain) to test it. Our citizen science study demonstrated that the nitrate concentration in the local tap water was approximately $100 \text{ mg}\cdot\text{L}^{-1}$. As a proof of concept, a volume of 3.5 mL of solution with an initial nitrate concentration of approximately $100 \text{ mg}\cdot\text{L}^{-1}$ was contacted with 1.6 mg of FNO_3 . After 2 hours, the nitrate concentration decreased to $67 \text{ mg}\cdot\text{L}^{-1}$, demonstrating effective adsorption. The material was then regenerated by immersion in a $500 \text{ mg}\cdot\text{L}^{-1}$ potassium chloride solution for 120 minutes and reused the following day in fresh Avellanosa del Páramo water with the same nitrate levels. This adsorption-regeneration cycle was repeated daily for a full week. As shown in Figure 3e, FNO_3 maintained its adsorption efficiency consistently across all seven cycles, confirming its stability and effective reusability under practical, user-friendly conditions.

However, this reuse study revealed a critical aspect that needed to be addressed: according to the adsorption equilibrium data presented in Table 1, the residual nitrate concentration in the water treated with FNO_3 should theoretically be approximately $47 \text{ mg}\cdot\text{L}^{-1}$, rather than the initially observed $67 \text{ mg}\cdot\text{L}^{-1}$. This discrepancy prompted the hypothesis that the nitrate removal efficiency of FNO_3 could be affected by the presence of competing anions in the water, particularly

carbonates and bicarbonates. These anions are commonly found in groundwater from calcareous regions and are known to interfere with anion exchange processes.

This observation also highlights that the potential use of this material by end users would necessarily need to be accompanied by an initial assessment of the water intended for purification. Such an assessment would allow the definition of critical operating parameters, including the immersion time required to reach a safe nitrate concentration, the number of uses possible before regeneration, and the number of regeneration cycles feasible before disposal. Importantly, all these parameters will strongly depend on the specific characteristics of each individual water source.

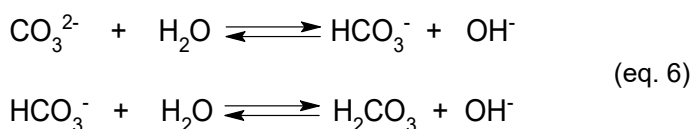
3.6.2. Interference and pH study

In this regard, a chemical analysis conducted by the company DXD Soluciones Químicas S.L. indicated that the water from the municipality of Avellanosa del Páramo exhibited a total alkalinity of $180 \text{ mg}\cdot\text{L}^{-1}$, suggesting a significant presence of carbonate and bicarbonate species. Given that these anions are common constituents of groundwater in regions with limestone geology—and are routinely included in standard water quality analyses—it was deemed appropriate to conduct a brief interference study to evaluate their impact on the nitrate removal performance of FNO_3 .

For this purpose, a series of sodium carbonate solutions were prepared at varying concentrations, ranging from 0 to $500 \text{ mg}\cdot\text{L}^{-1}$, all containing a fixed nitrate concentration of $95 \text{ mg}\cdot\text{L}^{-1}$. The experiments were conducted in Milli-Q water to eliminate background ionic interference. As shown in Figure 3f, an increase in carbonate concentration resulted in a progressively higher residual concentration of nitrates in the solution after treatment with FNO_3 . This trend clearly indicates that carbonate and/or bicarbonate ions act as significant interferents, competing with nitrates for the active adsorption sites in FNO_3 and thereby reducing its overall removal efficiency.

To better understand the role of carbonate interference in nitrate adsorption, it was necessary to consider the solution pH, as the carbonate/bicarbonate pair functions as a buffering system. Depending on the pH, the dominant species will be either carbonate (CO_3^{2-}) or bicarbonate (HCO_3^-). Therefore, both the initial and final pH values were measured in all sodium carbonate solutions used in the interference experiment, as shown in Figure 3f.

Carbonate originates from a weak acid, thus acting as a strong base that reacts with water to produce bicarbonate and hydroxide ions (OH^-). In turn, bicarbonate also reacts with water to generate carbonic acid and additional hydroxide ions, according to the following equilibrium, defined by Equation (6):



In this system, pH is crucial as it determines the concentration of each species at equilibrium. At the point with the lowest carbonate concentration (Figure 3f), the initial pH was 7.4 and increased to 8.1 after treatment with FNO_3 . Since adsorption is diffusion-controlled, higher concentrations correspond to a faster adsorption rate. At approximately pH 8, the dominant species is bicarbonate (>98%), which competes with nitrate for active sites, shifting the equilibrium toward increased formation of OH^- .

Interestingly, at higher carbonate concentrations, a slight decrease in the final pH is observed. This is because the carbonate, present in greater proportion, is also adsorbed alongside nitrate, shifting the equilibrium toward carbonate formation and thereby reducing the concentration of OH^- , which decreases the solution's basicity.

These observations also help explain the behaviour observed in the reuse assay with tap water from Avellanosa del Páramo, which had a pH of 7.8. Under these conditions, bicarbonate was the clearly predominant species

(>98%). This water exhibited an alkalinity of $180 \text{ mg}\cdot\text{L}^{-1}$ (expressed as CaCO_3), nearly twice the nitrate concentration. However, nitrate removal efficiency decreased by approximately 40%, indicating a higher selectivity of the FNO_3 material toward nitrates compared to bicarbonate. On the other hand, comparing the affinity for nitrate and carbonate is not pertinent in this case, as at $\text{pH} < 8.5$ the presence of carbonate anions in drinking water is practically negligible.

To further investigate the pH dependence of nitrate adsorption by FNO_3 , a series of controlled experiments were conducted with OH^- anions. A stock solution of nitrates ($92 \text{ mg}\cdot\text{L}^{-1}$) in distilled water with an initial pH of 6.24 was divided into five aliquots. Each aliquot was adjusted to a different initial pH by adding small volumes of NaOH, ensuring minimal dilution and maintaining consistent nitrate concentration across samples. Subsequently, 3.5 mL of each solution was treated with 1.63 mg of FNO_3 , and UV-Vis absorbance measurements were recorded at time zero and after 500 minutes. Final pH values were also monitored. To understand the intrinsic effect of FNO_3 on solution pH, the material was immersed in nitrate-free distilled water. The pH of this blank solution increased from 6.5 to 7.35, suggesting the release or uptake of species affecting proton concentration.

As shown in Figure 4, nitrate adsorption remained consistent across all tested pH values, indicating a broad operational range and robustness of the material's performance under varying acid-base conditions. Interestingly, in the most alkaline solution, a final pH of 7.49 was observed, slightly above the standard post-treatment value of 7.35. This behaviour suggests partial adsorption of OH^- ions in basic conditions, without significant impact on nitrate removal. Conversely, in more acidic media (first two solutions), where $[\text{H}^+] > [\text{OH}^-]$, the carbonyl groups within FNO_3 acted as proton acceptors, buffering the system and leading to a rise in final pH. This buffering capacity mimics that of a weak base and highlights the material's stabilizing effect on solution pH—particularly relevant for real-world water treatment scenarios where incoming pH conditions may vary. In future studies, the potential of this type of material as a solid-phase

pH buffering system (solid buffer) will be explored, given its ability to stabilize pH in solution.

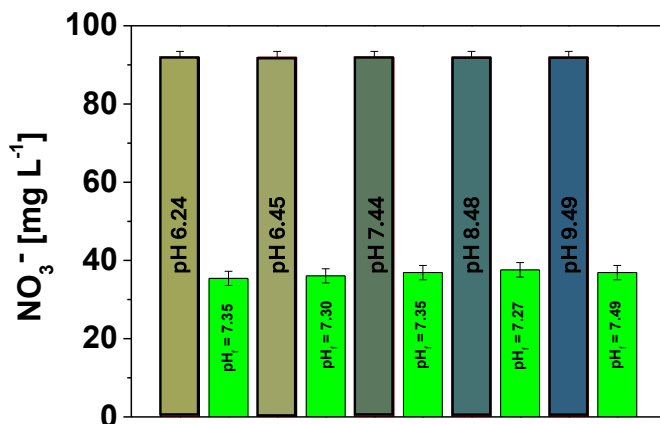


Figure 4. Influence of initial pH on the nitrate adsorption efficiency of the FNO₃ material. Solutions containing 92 mg·L⁻¹ of nitrates were adjusted to different pH values using NaOH and treated with 1.63 mg of material in 3.5 mL of solution. The bars show initial and final pH values and the amount of nitrate adsorbed after 500 minutes (light green). Results indicate that adsorption efficiency remains stable across the tested pH range, while the material exhibits a buffering effect that stabilizes the final pH around 7.35.

3.7. LCA

The environmental analysis of the nitrate removal film reveals a distinct distribution of impacts across its life cycle stages.

Production phase. The primary contributors to environmental impact are the production of glass (used as a structural material), transport of inputs, electricity consumption, and specific functional monomers (NNZA, VP, SA-1025) along with additives such as the photoinitiator DMPA and the crosslinker EGDMA. The impact of glass mainly arises from the extraction and processing of metallic and mineral resources, while transport and electricity impacts are largely due to fossil fuel use in energy generation and logistics. The functional monomers and additives significantly contribute to categories such as ecotoxicity, human toxicity, and resource use; however, their inclusion is justified by the functional value they provide to nitrate capture. The final FNO₃ film product itself accounts for a very

small fraction of the total impact, highlighting the system's functional efficiency (Figure 5a).

Impact weighting and improvement opportunities. Normalization results indicate that fossil resource depletion and non-carcinogenic human toxicity are priority areas for improvement, with carcinogenic human toxicity primarily linked to N-vinylpyrrolidone and NNZA (Figure 5b). Impact weighting based on the EF method identifies climate change and resource use as critical categories, largely due to material inputs and energy demands. Potential improvement strategies include substituting glass with bio-based or recycled materials, localizing supply chains, optimizing logistics, transitioning to renewable energy sources, and exploring more sustainable sourcing or reformulation of the monomers (Figures 5c and 5d). These findings should be interpreted considering the innovative nature of the system and its current laboratory-scale implementation, which has yet to benefit from industrial optimization.

Use phase. The use phase of the film exhibits a favourable environmental profile. Treated effluent can be valorised as an agricultural input, partially substituting conventional nitrogen fertilizers, which generates net benefits across aquatic ecotoxicity, human toxicity, eutrophication, and resource use categories. The potential for reusing the water or the film as a fertilizer will be addressed in future studies. The damage assessment confirms these positive trends, especially in resource use, human health, and ecosystem-related categories. While per-cubic-meter benefits may appear moderate on a per capita basis, their relevance increases in localized or decentralized contexts where closing water and nutrient loops is prioritized (Figures 5e and 5f).

Comparison with conventional technologies. Compared to nitrification-denitrification or anammox processes ³⁰, the film provides a more compact and versatile approach, with relatively lower impacts in some categories and higher in others, mainly due to specialized components. Compared to reverse osmosis or Ecogranularwater ³¹, the film exhibits higher impacts per

functional unit, attributable to its low TRL, laboratory-scale implementation, and lack of process optimization or input recovery.

3.8. Toxicology study. Determination of HepG2 cells response to the different water samples

Given that the primary route of exposure to drinking water is oral ingestion, a hepatic cell line was selected as the *in vitro* model, as the liver is the main organ responsible for the metabolism of ingested compounds and therefore represents a biologically relevant system for toxicity assessment. The viability of the human hepatocarcinoma cell line HepG2 after 24 hours of exposure to the highest tested concentration of water was evaluated using the *MTT assay*. This colorimetric test measures cellular metabolic activity based on the reduction of MTT to a purple formazan product by metabolically active cells, with absorbance measured at 590 nm. As shown in Figure 6, no substantial reduction in cell viability was observed in the water samples compared to the negative control. The *MTT assay* results indicate that 24-hour exposure of FNO₃ to the water samples did not induce cytotoxic effects.

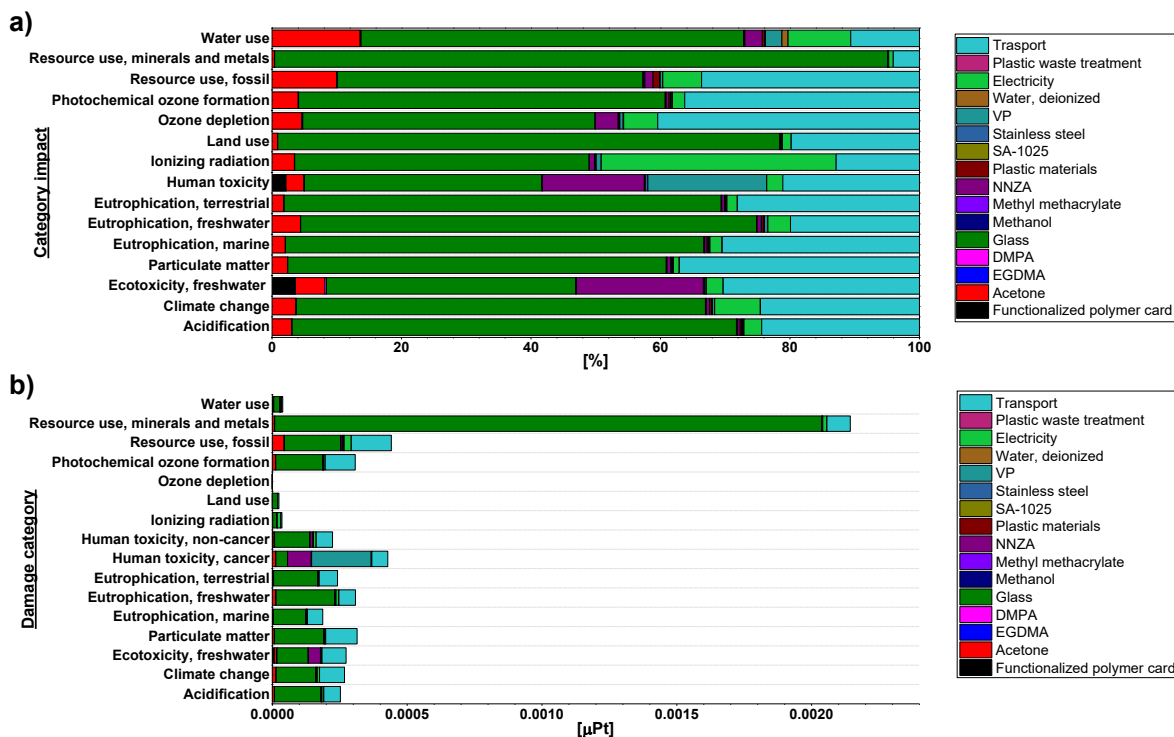


Figure 5. a) Percentage contribution of different processes and materials to the environmental impact by category (characterization) during the production phase of the functional film for nitrate removal. b) Normalized environmental impact by category associated with the production of the functional film for nitrate removal. This diagram provides a relative comparison among impact categories according to the Environmental Footprint 3.1 method.

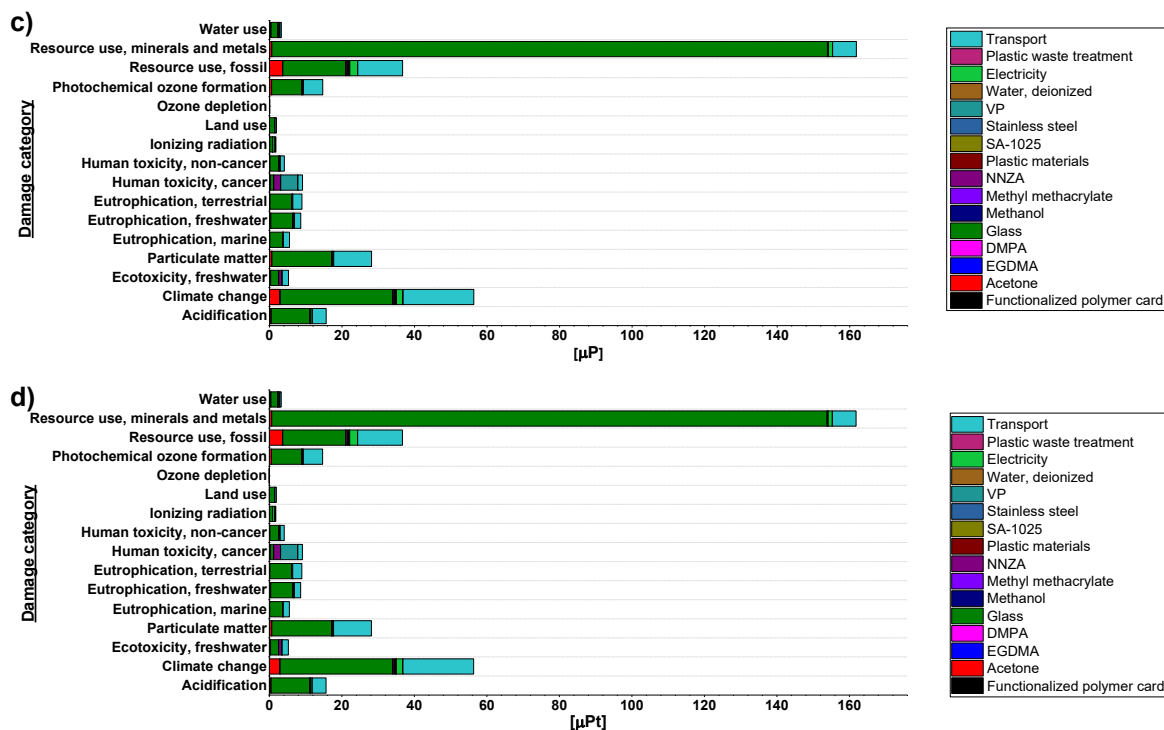


Figure 5. c) Weighted environmental impact by category for the production of the functional film for nitrate removal, based on the Environmental Footprint 3.1 method. d) Contribution of each process to the total environmental impact (aggregated single score) during the production of the functional film for nitrate removal, as assessed using the Environmental Footprint 3.1 method.

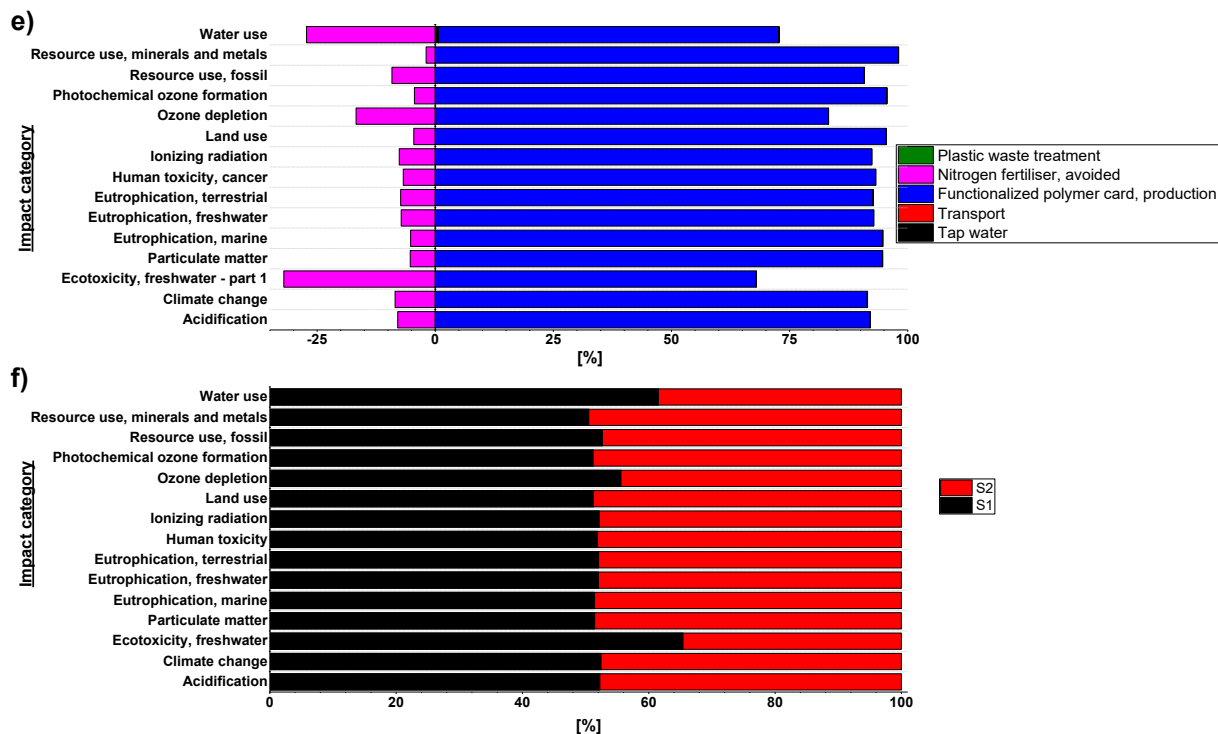


Figure 5 e) Characterization of the environmental impact by category of the processes involved during the use phase of the functional film for nitrate removal, according to the Environmental Footprint 3.1 method. f) Comparison of the environmental footprint profile between two use scenarios of the film: S1 (without valorization of the regenerated water) and S2 (with valorization as fertilizer), expressed as the percentage of net impact per category.

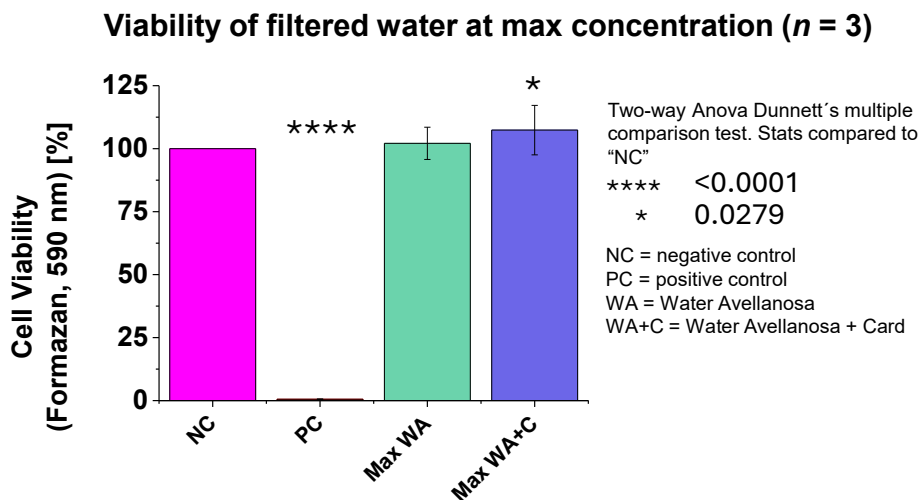


Figure 6. a) Viability of HepG2 cells treated with different water samples at their maximum concentration. Results are expressed as a percentage of negative control (untreated cells). Data represent the *mean* ($\pm$ *standard deviation*, *SD*) of 3 biological replicates with 4 technical replicates. Differences are established using a *Two-way ANOVA* followed by a multicomparison test (*Dunnett's test*), and considered significant when $P \leq 0.05$.

4. Practical Applications and Future Research Prospects

The multifunctional FNO_3 polymeric thin films developed in this work have clear practical applications in both water treatment and environmental monitoring. Their high selectivity and reusability make them suitable for decentralized nitrate removal in small-scale or rural water systems, where conventional technologies such as reverse osmosis or ion-exchange resins are inefficient, wasteful, or cost-prohibitive. The integrated fluorescence signal provides a simple visual cue for saturation, enabling non-specialized personnel and community members to monitor water quality effectively. This combination of curative and sensory functionality supports citizen science initiatives, allowing local communities to participate in environmental monitoring and fostering awareness of nitrate pollution and preventive actions.

Future research should focus on several aspects to enhance the material's applicability and performance. First, detection accuracy and precision

under a wider range of environmental conditions should be assessed. Second, interference studies should be expanded to include diverse ions and complex water matrices commonly found in natural and agricultural settings. Third, scaling up production while maintaining material quality and cost-effectiveness will be essential for practical deployment. Additionally, long-term stability, repeated reuse, and integration with circular recovery strategies for captured nitrates should be investigated. Finally, further life cycle assessments and toxicological evaluations will help ensure environmental safety and support industrial-scale implementation.

5. Conclusions

This work presents a comprehensive approach to nitrate pollution by integrating multifunctional polymeric thin films (FNO₃) with citizen science initiatives. FNO₃ combines nitrate removal and visual detection via fluorescence, demonstrating high selectivity, reusability, and practical limits of detection for real-world applications. Citizen participation revealed strong engagement, highlighting public demand for information and preventive action. Field studies showed site-specific nitrate variations, emphasizing the need for tailored interventions. Preliminary Life Cycle Assessment and toxicological evaluations confirmed the material's environmental safety and scalability. Overall, this approach provides a low-cost, decentralized, and circular solution to nitrate pollution, empowering communities, promoting awareness, and supporting environmental and economic resilience. Future work should refine detection accuracy, expand interference studies, and optimize industrial-scale production, advancing FNO₃ toward practical deployment in water treatment.

Data Availability

Open Data is available at <https://riubu.ubu.es/handle/10259/5684> under the name "UBU-Polymers Research Group 04072025".

Competing interests

The authors declare no competing interests.

Acknowledgments

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Supplementary Material

Results of the citizen science project; quantification of nitrate in water samples; synthesis of the polymer FNO3 and characterization by FTIR, TGA and DSC; synthesis of the sensory monomer SA-R1025; sorption study.

The Supplementary Material is available free of charge at:

<https://ars.els-cdn.com/content/image/1-s2.0-S2352186425005814-mmc1.pdf>

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CONCLUSIONES

Como conclusión general, se puede afirmar que, en este trabajo, ha quedado demostrado que los polímeros inteligentes en formato film presentan un gran potencial para su aplicación como sensores o catalizadores en diferentes sectores, gracias, entre otros factores, a su versatilidad química, su formato robusto y su bajo coste. Las diferentes investigaciones llevadas a cabo en esta tesis han permitido diseñar, desarrollar y aplicar varios polímeros reactivos (en combinación con la inmovilización de enzimas) y sensores a lo largo de la misma, lo que ha posibilitado una mayor comprensión y validación de estos polímeros inteligentes.

Como conclusiones específicas, se pueden establecer las siguientes:

- Se ha demostrado la posibilidad de utilizar los films poliméricos en la industria alimentaria:
 - Concretamente, en la industria láctea, se ha desarrollado un polímero reactivo, en combinación con la tecnología de inmovilización de enzimas, para la hidrólisis de lactosa mediante la enzima β -galactosidasa anclada covalentemente al film, el cual es reutilizable. Por otro lado, una variante de la misma matriz polimérica se ha aplicado como sensor colorimétrico, capaz de cuantificar proteínas en suero lácteo y sus concentrados de manera alternativa y más eficiente que los métodos actuales.
 - Paralelamente, se han obtenido dos films poliméricos sensores para la cuantificación de betalaínas y del poder antioxidante en remolachas, de manera sencilla, rápida y sin equipamiento costoso.
 - Se ha demostrado la posibilidad de aplicar los films poliméricos en remediación ambiental:
-

- Nuevamente, en combinación con la inmovilización enzimática covalente, se ha obtenido un polímero reactivo con la enzima lacasa anclada, el cual ha permitido la degradación de bisfenol A e índigo carmín en aguas.
- Finalmente, se ha desarrollado un film polimérico con doble función, capaz de captar y detectar nitratos en agua, todo ello pensando en la simplicidad para el usuario final.

Los resultados obtenidos en esta tesis doctoral abren un nuevo abanico de posibilidades para los films poliméricos inteligentes, tanto como sensores como catalizadores. Su versatilidad química, combinada con la amplia variedad de enzimas disponibles, multiplica el rango de aplicaciones de estos novedosos materiales de cara al futuro, no solo en las áreas abordadas en este trabajo, sino también en otras, como la medicina o la ingeniería, convirtiéndolos así en una tecnología prometedora y con un gran potencial de investigación.

CONCLUSIONS

As a general conclusion, it can be stated that this work has demonstrated that smart polymers in film format exhibit great potential for their application as sensors or catalysts in different sectors, owing, among other factors, to their chemical versatility, robust format, and low cost. The various investigations carried out throughout this thesis have enabled the design, development, and application of several reactive polymers (in combination with enzyme immobilization) and sensors, thereby allowing a deeper understanding and validation of these smart polymers.

The following specific conclusions can be drawnThe feasibility of using polymeric films in the food industry has been demonstrated:

- Specifically, in the dairy industry, a reactive polymer has been developed, in combination with enzyme immobilization technology, for lactose hydrolysis using the enzyme β -galactosidase covalently anchored to the film, which is reusable. In addition, a variant of the same polymeric matrix has been applied as a colorimetric sensor capable of quantifying proteins in whey and whey concentrates in an alternative and more efficient manner than current methods.
- In parallel, two polymeric sensor films have been obtained for the quantification of betalains and antioxidant capacity in beetroot, in a simple, rapid, and cost-effective manner.
- The feasibility of applying polymeric films in environmental remediation has been demonstrated:
 - Once again, in combination with covalent enzyme immobilization, a reactive polymer with anchored laccase enzyme has been obtained, which has enabled the degradation of bisphenol A and indigo carmine in water.
 - Finally, a polymeric film with dual functionality has been developed, capable of both capturing and detecting nitrates in water, with particular emphasis on ease of use for the end user.

The results obtained in this doctoral thesis open up a new range of possibilities for smart polymeric films, both as sensors and as catalysts. Their chemical versatility, combined with the wide variety of available enzymes, broadens the range of potential applications of these novel materials in the future, not only in the areas addressed in this work, but also in others such as medicine and engineering, thus positioning them as a promising technology with significant research potential.

APPENDIX

1. Patent:

Colorimetric dual-range polymeric sensor for rapid and direct quantification of protein concentration in serum and commercial whey protein concentrates (WPC).

2. Book chapter:

Vallejo-García JL, Cutillo-Foraster A, Arnaiz A, et al. Hydrolysis of lactose: conventional techniques and enzyme immobilization strategies on polymeric supports. in: milk proteins - Technological Innovations, Nutrition, Sustainability and Novel Applications. *IntechOpen*; 2024. doi:10.5772/intechopen.1005087.

1.



Solicitud de patente nacional

Información de solicitud

Referencia de usuario

Ref.2026.03 Suero Lácteo

ID de la solicitud

0000054132

54 Título de la invención

Sensor polimérico colorimétrico de doble rango para cuantificación rápida y directa de la concentración proteica en suero y concentrados comerciales de suero lácteo (WPC)

Partes intervinientes

71 Solicitante 1

Nacionalidad

España

Categoría

Universidad pública española

Nombre de la organización

Universidad de Burgos

Departamento

OTRI

NIF

Q0968272E

País

España

2.

 OPEN ACCESS PEER-REVIEWED CHAPTER

Hydrolysis of Lactose: Conventional Techniques and Enzyme Immobilization Strategies on Polymeric Supports

WRITTEN BY

Jorge Lucas Vallejo-García, Alessandra Cutillo-Foraster, Ana Arnaiz, Saúl Vallejos, José M. García, María Asunción Muñoz Santamaría and Miriam Trigo-López

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Abstract

This chapter explores lactose hydrolysis, emphasizing conventional techniques and the noteworthy immobilization of β -galactosidase on polymeric matrices to enhance the process. Lactose, present in milk and dairy, poses challenges for lactose-intolerant individuals, requiring enzymatic hydrolysis for lactose-free product development. The presence of other milk components, such as proteins and minerals, can indirectly influence the efficiency of lactose hydrolysis by potentially interacting with β -galactosidase enzyme or affecting its stability and activity, making it necessary to control factors such as enzyme concentration, temperature, pH, and reaction time to improve lactose hydrolysis rates. The chapter delves into established methodologies, covering enzymatic kinetics, reaction conditions, and substrate concentrations. It also describes the innovative approach of immobilizing β -galactosidase on polymeric supports to enhance enzyme stability, reusability, and overall efficiency in lactose hydrolysis. Discussions include the design of suitable polymeric matrices, providing insights into mechanisms governing catalytic performance. This comprehensive exploration contributes to understanding lactose hydrolysis, offering valuable insights for developing efficient and sustainable enzymatic processes applicable to the food and pharmaceutical industries.

Keywords

dairy products

lactose hydrolysis

enzyme immobilization

advanced polymers

polymeric supports