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Extraction of plant biopolymers and their use in gas sensitive hybrid materials

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Extraction of plant biopolymers and their use in gas sensitive hybrid materials

"Lançam-te para um mundo estranho onde não queres entrar,

nasces e amas, depois perdes e choras

e no fim partes a querer ficar"

Carolina Tendon

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RESUMO

Os biopolímeros têm estimulado o interesse devido às suas excelentes propriedades, tais como impermeabilização, atividade antimicrobiana e elevada versatilidade, bem como a capacidade de formarem materiais híbridos com propriedades sensitivas a estímulos.

Com esta finalidade, extrações de suberina da cortiça (*Quercus suber*) e da pele de batata branca (*Solanum tuberosum L. CV. Monalisa*) e de cutina da pele do tomate (*Solanum lycopersicum*) foram realizadas através de um processo inovativo baseado na capacidade do líquido iónico Cholinium Hexanoate de clivar seletivamente as ligações acylglycerol do tipo éster, permitindo a recuperação parcialmente intacta dos polímeros mencionados. A extração de suberina de cortiça resultou em rendimentos de ≈2-20%, usando períodos de extração de 30 minutos, 1 hora e 2 horas, enquanto a extração de 2 horas de cascas de batata branca renderam ≈4% de suberina e as extrações de 2 horas de pele de tomate sem tratamento e enzimaticamente digerido rendeu ≈6% e ≈1% de cutina, respetivamente.

A aplicação dos biopolímeros extraídos em materiais sensitivos para um nariz eletrónico (E-Nose) em desenvolvimento foi testada combinando a suberina e a cutina com água e cristal líquido 4-cyano-4-pentylbyphenil (5CB). Os materiais híbridos obtidos foram processados como filmes finos e posteriormente expostos a cinco compostos orgânicos voláteis (COVs) com diferentes polaridades – hexano, tolueno, diclorometano, etanol e acetona. Uma resposta óptica foi registada após exposição aos COVs e os resultados revelaram uma tendência dos filmes baseados em suberina para respostas ópticas mais elevadas após exposição a tolueno, diclorometano e acetona e manutenção do seu rendimento e morfologia, enquanto os filmes baseados em cutina se desintegraram após a exposição ao tolueno e revelaram tendência para respostas ópticas mais baixas para qualquer um dos voláteis. Nenhum filme desencadeou uma resposta óptica na presença de etanol ou hexano no E-Nose.

Estes resultados reforçam o interesse na exploração de biopolímeros de plantas, em especial a suberina, como componentes valiosos para a produção de materiais híbridos com propriedades sensitivas a estímulos.

Palavras-chave: biopolímero; líquido iónico; cristal líquido; sensor de gás; compostos orgânicos voláteis.

ABSTRACT

Biopolymers have spurred interest because of their outstanding properties, such as waterproofing, antimicrobial activity and resistance, as well as the ability to help form hybrid materials with stimuli-responsive properties.

With this purpose, extractions of suberin from cork (*Quercus suber*) and white potato skin (*Solanum tuberosum L. cv. Monalisa*), and cutin from tomato skin (Solanum lycopersicum) have been achieved through a novel process based on cholinium hexanoate's selective cleavage of acylglycerol ester bonds, allowing the partially intact recovery of the biopolymers mentioned. The extraction of suberin from cork resulted in yields ranging from ≈2-20%, using extraction periods of 30 minutes, 1 hour and 2 hours, while 2-hour extraction from white potato peels yielded ≈4% suberin and 2-hour extraction from both natural and enzymatically digested tomato skin yielded ≈6% and ≈1% cutin, respectively.

The application of the extracted biopolymers as sensing materials for an electronic nose (E-Nose) under development was tested by combining the biopolymers suberin and cutin with water and the liquid crystal 2-cyano-4-pentylbiphenyl (5CB). The hybrid materials obtained were processed as thin films and further exposed to five volatile organic compounds (VOCs) with different polarities – hexane, toluene, dichloromethane, ethanol and acetone. An optical response was recorded upon VOC exposure and the results revealed a tendency from suberin-based films to higher optical responses to toluene, dichloromethane and acetone and maintenance of their yield and morphology after exposure, while cutin-based films disintegrated after exposure to toluene and revealed much lower optical responses to all VOCs. No film gave an optical signal in the presence of ethanol or hexane in the E-Nose.

These findings strengthen the interest in exploiting plant biopolymers, specifically the polyester suberin, as valuable components for the production of hybrid materials with stimuli responsive properties.

Keywords: biopolymer; ionic liquid; liquid crystal; gas sensing; volatile organic compound.

ABBREVIATIONS

5CB – 4-cyano-4-pentylbiphenyl

AFM – Atomic Force Microscopy

ATR FT-IR – Attenuated Total Reflectance Fourier-Transform Infrared Spectroscopy

[BMIM][DCA] – 1-butyl-3-methylimidazolium dicyanamide

DCM – Dichloromethane

E-NOSE – Electronic nose

GC-MS – Gas Chromatography-Mass Spectrometry

HPLC – High-Performance Liquid Chromatography

LAW – Love Acoustic Wave

LC – Liquid crystal

PCA – Principal Component Analysis

POM – Polarized Optical Microscopy

SAW – Surface Acoustic Wave

SEM – Scanning Electron Microscopy

STD.DV – Standard Deviation

VOC – Volatile Organic Compound

VP – Vapour Pressure

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LITERATURE REVIEW

Suberin and Cutin biopolymers

Biopolymers are biological-based compounds produced by living organisms. Most of them represent viable alternative to petroleum-based polymers and are, as such, objects of great interest when the focus is environmental sustainability. One such biopolymer is suberin, a complex hydrophobic polyester ubiquitously found in nature, of high abundance in the periderm of *Quercus suber* (H. Pereira, 1988), underground organs such as the tubers of *Solanum tuberosum* and also in the endodermis of roots (Gandini, Pascoal Neto, & Silvestre, 2006). Arising under the form of a thick-lamellar layer in plant cell walls during cell differentiation processes (Mattinen et al., 2009), suberin's main role is the protection of the plant against pathogens and external physical aggressions (Ferreira et al., 2014) which in turn further stimulate its production (Sidibé et al., 2016). While not totally deciphered, suberin is known to be a structure of aliphatic and aromatic monomers, highly cross-linked via ester bonds with glycerol molecules, comprising a macromolecular structure with high abundance in C20-C24 fatty acids (Li-Beisson, 2011) and showing a high resistance to enzymatic digestions and chemical treatments (Mattinen et al., 2009). An increased interested in this polymer rises from the fact that it is a rich source of ω-hydroxyacids and epoxy-fatty acids with midchain functionalities of useful impact in the production of novel materials (Ferreira et al., 2013). This interest led to extensive analysis of the monomeric composition of suberin along decades, especially through gas-chromatography coupled with mass spectrometry (GC-MS), revealing a high abundance in alkanoic acids, α,ω-alkanedioic acids, ω-hydroxyalkanoic acids, dihydroxyacids, trihydroxy-acids, epoxyacids and 1-alkanols (Lopes, Gil, Silvestre, & Neto, 2000), with 22-hydroxydocosanoic acid, 18-hydroxyoctadec-9-enoic acid and 9,10-dihydroxyoctadecanedioic acid as the major contributors (Lopes et al., 2000)(Ferreira et al., 2014).

Another highly regarded polymer is cutin. It differs from suberin first by being part of the cuticle, a lipidic membrane that covers the epidermal cell walls of aerial plant organs (Lara, Belge, & Goulao, 2015) such as the fruits of *Solanum lycopersicum* and other angiosperm plant leaves. While also playing a protective role, the cuticular layer is also vital for the dynamic relation with the environment, and cutin is known to be one of its major components (Martin & Rose, 2014). Like suberin, cutin is a glycerolipid polymer but with high abundance in hydroxylated and epoxy-hydroxylated C16-C18 fatty acids (Lara et al., 2015). Its monomeric composition varies among botanical species, but experiments leading to complete depolymerization of cutin with consequent GC-MS analysis shows that ω-hydroxyacids comprise a majority of the polymer, with the main representatives being 16-hydroxyhexadecanoic acid, 10,16-dihydroxyhexadecanoic acid and 9,10-epoxy-18-hydroxyoctadecanoic acid.

Extraction methods

Insoluble biopolymer extraction from natural resources is usually achieved through alkaline methanolysis or aqueous or alcoholic alkaline hydrolysis. The first is the most utilized and can be done with the methanolic-hydrogen chloride solution (MeOH-HCl), boron trifluoride-methanol solution (BF3-MeOH) or methanolic sodium methoxide (NaOMe), among others, but it is usually done with the latter. This process leads to a complete depolymerization of the polymer while being the least harsh method to determine the full monomeric composition of suberin (Gandini et al., 2006) because it allows detection of epoxy moieties indirectly in the form of methoxyhydrins (Lopes et al., 2000), while aqueous alcoholic conditions in alkaline hydrolysis can lead to unstable depolymerizations and release of interfering depolymerization compounds, such as high amounts of polyphenols. Another method tested on both cutin and suberin depolymerization was hydrogenolysis with $LiAlH_4$ in tetrahydrofuran, but these tests showed that distinction of epoxide and carbonyl groups is lost with this method, which can be solved using deuterolysis with $LiAlD_4$ in tetrahydrofuran followed by gas-liquid chromatography coupled with mass spectrometry GLC-MS (Walton & Kolattukudy, 1972) or recurring to BF3-MeOH transesterification for a more adequate depolymerization (Kolattukudy & Agrawal, 1974).

Nonetheless, the methods described above result in an extensive depolymerized biopolymer leading to the loss of its properties. As such, an attempt to simplify the process while obtaining a partially intact polymer resulted in a novel method based on the dissolution in biocompatible ionic liquids (Garcia et al., 2010). These first tests were performed on refined cork (i.e. free from extractives) due to its resemblance with lignocellulosic materials, hence, being potentially soluble in tailor-made ionic liquids. The best results, where Fourier transform-infrared spectroscopy (ATR FT-IR) revealed that polymer-related peaks were greatly reduced in refined cork after dissolution in ionic liquid, were obtained with cholinium-based ionic liquids, more specifically with cholinium hexanoate ($[N_{111}C_2H_4OH][O_2CC_5H_{11}]$). Besides yielding a moderately cross-linked suberin, capable of self-assembly and antimicrobial effects, cholinium hexanoate is a biodegradable and biocompatible ionic liquid that can be recycled and reused after extraction (Petkovic et al., 2010). The reason for this mild depolymerization lies on the selectivity of cholinium hexanoate towards cleaving acylglycerol ester bonds, while preserving linear aliphatic ester bonds of the biopolymer (Ferreira et al., 2014). After depolymerization, the recovery of the polymer is achieved through simple precipitation in water, and the formation of self-assembled films is made possible with slow evaporation of water with a suspension of suberin on a polystyrene plate (Garcia et al., 2014).

Thanks to this preservation of important characteristics such as antibiofouling and antimicrobial activities and moderate hydrophobicity, suberin and cutin resulting from this innovative method can both be used for the production of new materials in film form, namely for medical applications such as grafts and implant packaging, or even cosmetic applications.

Gas Sensing Materials and the Electronic Nose

A gas sensor is a chemical sensor that responds the presence of gas molecules or a volatile organic compound (VOC), and transforms it into an electrical signal. Gas sensing technology has a wide range of applicability, whether for detection of pollutants in the environment or industrial sites (e.g. greenhouse or mine-related gas detection), for indoor air and food quality control systems (e.g. carbon-monoxide contamination and food spoilage control, respectively) or for medical applications, such as the detection of lung cancer through breath analysis (James, Scott, Ali, & O'Hare, 2005). This variety of applications resulted in the production of various types of gas sensors, which implies an even greater amount of gas sensitive materials with different sensitivity and selectivity. Metal oxide sensors, for example, rely on conducting and semiconducting metal oxides such as TiO_2, SnO_2 and ZnO as means for detecting explosive gases (Meixner & Lampe, 1996). Others like acoustic wave sensors (Surface Acoustic Wave (SAW) and Love Acoustic Wave (LAW) sensors) take advantage of the properties of piezoelectric materials (Bo et al., 2016) for chemical warfare agents, and optical fibers find usefulness in polymer-coating for detection of nerve agents and VOCs (Aernecke & Walt, 2009).

Over the past few years, with the growing need to improve people's quality of life, be it through food monitoring, disease prevention and control or environmental protection, society increasingly demonstrates the will to overcome these problems with cost-effectiveness, miniaturization and high sensitivity of gas sensors, which led to the development of the Electronic Nose (E-Nose). This device mimics the olfactory system of the human being (Gardner & Bartlett, 1994) and requires an array of independent semi-selective gas sensors, a signal transduction unit followed by a data analysis and pattern recognition software to work (James et al., 2005). One of the goals of this device is to detect patterns of VOCs that are closely linked to the presence of harmful microorganisms, such as the ones causing respiratory infections or food spoilage.

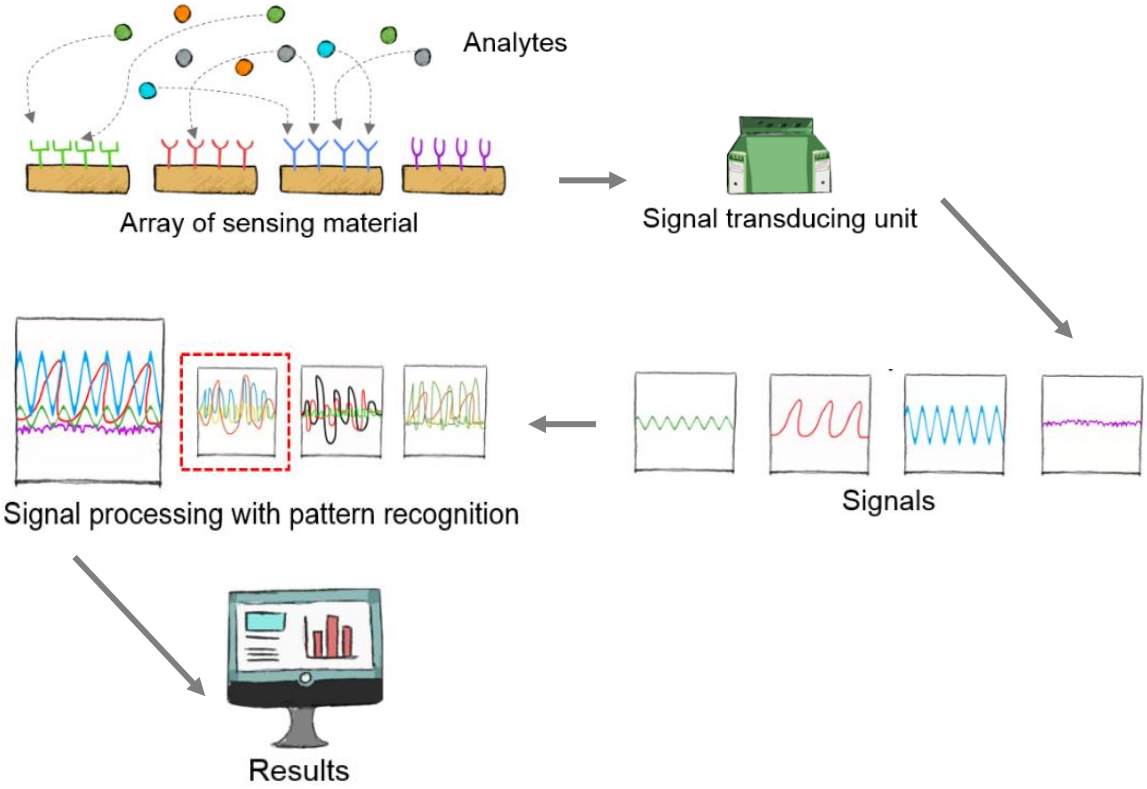

Figure 1 – Schematic representation of the steps that comprise the E-Nose sensing system.

Liquid Crystals in Gas Sensing

Liquid crystal (LC) is a state of matter where the arrangement is like a solid, but flows like a liquid. They can self-arrange in different mesophases, highly sensitive to external stimuli, such as an electrical or magnetic field (A. Hussain et al, 2009), radiation or humidity (Carlton et al., 2013). These properties allow for molecular events to be amplified in surfaces with anchored LCs, making them responsive materials. For several decades, LCs have been exploited for this potential, with LCDs being the best example of their popularity: an electrical field is applied to the LC, causing it to change its direction and hence, produce an optical response (Carlton et al., 2013). More recently, this property of LCs to change their orientation when exposed to stimuli has further encouraged their incorporation in gas sensing materials. Research has shown that LCs can respond to organic vapors by optical reflection (Poziomek, Novak, & Mackay, 1974).

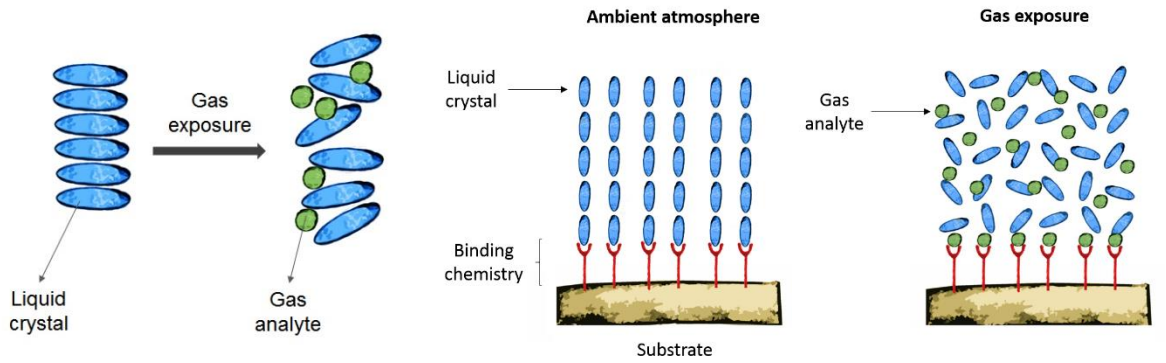

Figure 2 – Examples of the mechanisms for LC-based gas Sensing.

The sensing mechanism is based on the perturbation of the organized LC when the gas molecules interact with the anchoring surface, or LC film (Figure 2), changing the optical pattern of the LC that can be visualized under polarized optical microscopy (POM). This prompts the idea of developing hybrid materials incorporating LCs. One example of such successful incorporation was demonstrated (A. Hussain et al., 2017) and relied on the encapsulation of cholesteric liquid crystal with ionic liquid (IL), forming LC-IL droplets and stabilizing them in a biopolymer matrix. These materials, called hybrid gels, were then inserted between two crossed polarizers and were proven to respond to chemical, physical, and mechanical stimuli, resulting in optical and electrical signals.

RESEARCH STRATEGY

The objectives of this work were to extract the biopolyesters suberin and cutin from refined cork, tomato skin and white potato skin using a novel process developed by the Applied and Environmental Mycology group (AEM) at ITQB-NOVA that relies on the selective cleavage of acylglycerol ester bonds in the biopolyester by the ionic liquid cholinium hexanoate. After extraction, the purpose was to produce hybrid materials incorporating liquid crystal (LC) as an optical probe into a matrix of the biopolyesters as to verify the optical response of these materials in the presence of volatile organic compounds (VOCs) in an electronic nose (E-Nose) under development at the Biomolecular Engineering Lab at FCT-NOVA. The overall research strategy of the work is present in Figure 3.

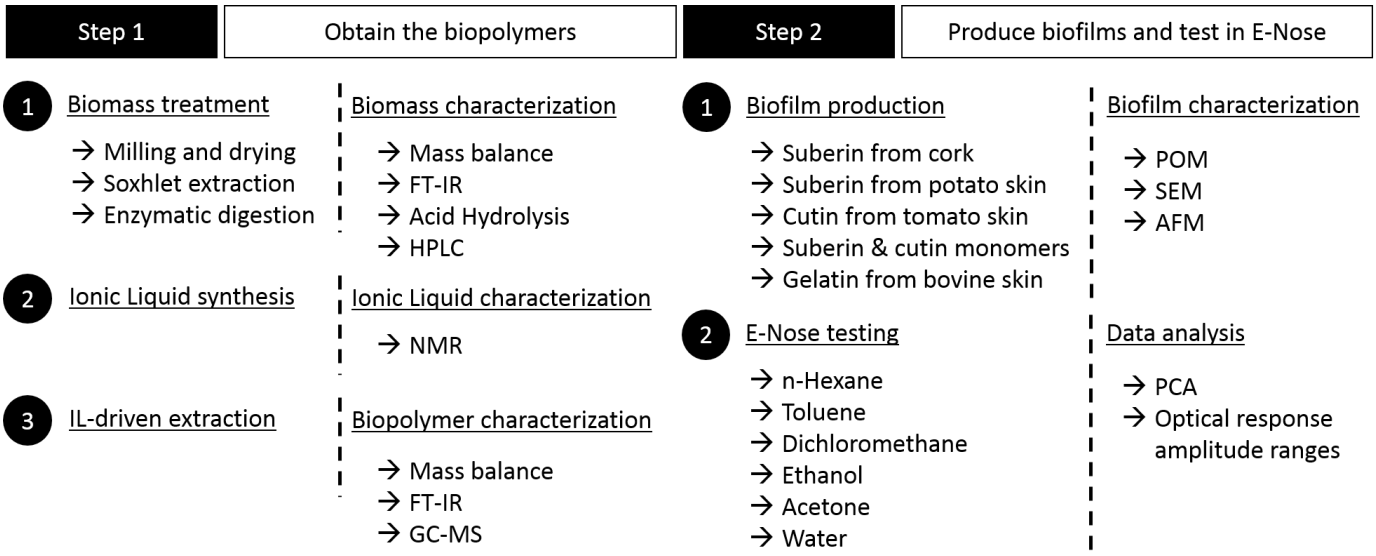

Figure 3 – Research strategy.

The novelty of this work relies on testing the viability of the unexplored application of suberin and cutin biopolymers obtained through the method aforementioned to produce hybrid materials with stimuli responsive properties.

CHAPTER 1 | Biopolymer attainment

1.1 Introduction

In this first chapter, the results and discussion relative to the extraction of suberin from refined cork (*Quercus suber)* and white potato skin (*Solanum tuberosum L. cv. Monalisa*), and cutin from tomato skin (*Solanum lycopersicum*) using the already described novel method developed by the AEM group at ITQB-NOVA are presented. These include ATR FT-IR, HPLC and mass balance results from enzymatic digestions performed on white potato and tomato skin, mass balance and polymer yields of 30-minute, 1-hour and 2-hour extractions of suberin from cork, and 2-hour extractions of suberin from white potato skin and cutin from tomato skin and biomass/biopolymer characterization through ATR FT-IR, HPLC and GC-MS. The treatment of the biological material with Soxhlet extractions or enzymatic hydrolysis before extracting the biopolymer had the purpose of obtaining a "cleaner" and extractive-free material or a polysaccharide-free material, respectively, possibly translating in the recovery of higher purity biopolyesters from the treated source. While the purpose of the work was to ultimately extract suberin and cutin from both treated and untreated natural sources to compare extraction yields and the feasibility of the ensuing products, only the latter was extracted from treated and untreated tomato skin, whereas suberin was extracted from untreated white potato skin and refined cork.

1.2 Materials and Methods

Biomass

Three types of biological material were utilized for the extraction of biopolymers – cork (*Quercus suber*), white potato skin (*Solanum tuberosum L. cv. Monalisa)* and tomato peels (*Solanum lycopersicum*). Cork was obtained from the producers Amorim & Irmãos SA (S^{ta} Maria de Lamas, Portugal), white potatoes were bought in a local supermarket and come from the producers Batatas Mirense, Lda. (Mira, Portugal) and tomatoes were obtained from a commercial supplier in Oeiras. White potato peels were obtained by peeling the raw tubers and the peels were then boiled in water for 5 minutes. They were then scrapped to assure minimum pulp content. The scrapped peels were first air dried in ambient temperature and then at 50^{o}C in a heater until constant weight was reached (usually a few hours). After drying, the peels were milled using a Retsch ZM200 electric grinder (granulometry 0.5mm; 10000rpm) and stocked on plastic bottles and falcons at ambient temperature. Tomato peels and cork were already available in the AEM Laboratory.

Ionic liquid (IL)

Cholinium Hexanoate (Figure 1.1) was synthesized by dropwise addition of Hexanoic Acid (Sigma Aldrich, >99.5%) on Choline Bicarbonate (Sigma Aldrich, ~80% in H_2O), while stirring. In the end, cholinium hexanoate was washed with diethyl ether (Sigma Aldrich) and left overnight for phase separation. IL purity was verified through ^{1}H-NMR using a Bruker 500 spectrometer with D_2O as solvent (Figure 1.21 in Appendix). For the drying of the ionic liquid, a rotavapor was used for water evaporation (35-40°C; 80-10^{-3}mbar) and a lyophilizer for water sublimation. The water content in cholinium hexanoate was determined through Karl-Fisher titration and values were between 0.7-1.8%.

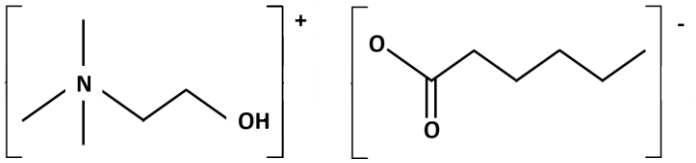

Figure 1.1 – Molecular structure of Cholinium Hexanoate.

Reagents and enzymes for enzymatic digestion

Acetate buffer pH4 was synthesized with acetic acid and sodium acetate bought from Sigma- Aldrich. The enzymes used were cellulase from *Trichoderma reesei* (5g/L; d=1.2g/mL; 700U/mg) and pectinase from *Aspergillus aculeatus* (1g/L; d=1.16g/mL; >3.800U/mL), both bought from Sigma-Aldrich.

Other chemicals

Dichloromethane (>98%), ethanol (absolute) sulphuric acid (>96%) and phenol (>99%) were purchased from Sigma-Aldrich.

Soxhlet extractions

Soxhlet extractions were applied with the purpose of removing the extractable fraction and followed a method previously described (Ferreira et al., 2014). Briefly, ≈4g of untreated biological material, already dried and milled, was weighted in a cellulose cartridge, covered with cotton. The cartridge was stapled to avoid contamination and loss of material. The extractable fraction was removed by sequential extractions with solvents of increasing polarity: dichloromethane (8h), ethanol (8h) and distilled water (24h). After extraction, the biomass was

recovered, washed with distilled water for 30 minutes at 100°C, under agitation of 800 rpm and lyophilized.

Enzymatic digestion

After Soxhlet extractions, the biological materials were submitted to enzymatic hydrolysis for the removal of polysaccharides. The enzymatic hydrolysis used in this study was a modified version of a method already described (Walton & Kolattukudy, 1972). In brief, a solution of Acetate buffer pH4, containing cellulase from *Trichoderma reesei* (5g/L; d=1.2g/mL; 700U/mg) and pectinase from *Aspergillus aculeatus* (1g/L; d=1.16g/mL; >3.800U/mL) was applied to different amounts of biological material post-Soxhlet extraction and left for 14h at 30°C with 300rpm of agitation. Three enzymatic ratios were tested, where the variable was biomass content, and were planned as follows:

- 0.51g cellulase:0.10g pectinase per 1g biomass: 0.050g of biomass was weighted in a test-tube. 5mL of enzymatic solution (21µL of cellulase + 4,3µL of pectinase + Acetate Buffer pH4) was added to the test-tube.
- 0.97g cellulase:0.19g pectinase per 1g biomass: 0.0260gr of biomass was weighted in a test tube. Everything else was the same as with the previous ratio.
- 3.17g cellulase:0.63g pectinase per 1g biomass: 0.008g of biomass was weighted in a test tube. Everything else was the same as with the previous ratio.

All tests were implemented in triplicate and controls were made using 5mL of the Acetate Buffer without enzyme. After each hydrolysis, the solutions were filtered through a membrane (Pall Life Sciences; Supor®200 47mm 0.2µm), previously weighted, through vacuum, with addition of 5mL of Acetate Buffer pH4 and at least 20mL of distilled water. The membranes were lyophilized and weighted the next day. Mass balance was made to allow calculations of loss biomass (%) for each enzyme ratio and each source. Before proceeding with the extraction, a bibliographic search was made on the polysaccharidic content in each source to allow a more complete data analysis and the results are shown in Table 1.1 below.

Table 1.1 – Polyester, lipid, polysaccharide and protein content in each of the three sources used.

	% polyester	% lipids	% polysaccharides	% protein
Potato skin	24.6[(1)]	0.6[(2)**]	30.4%[(7)*]	15.3[(2)**]
Tomato skin	985ug/cm^2[(8)]	4.04[(4)]	30*[(5)]	10.7[(3)]
Cork	50[(6)]	-	20[(6)]	-

(1) (Graça & Pereira, 2000); (2) (Schieber & Saldaña, 2009); (3) (Al-Wandawi, Abdul-Rahman, & Al-Shaikhly, 1985); (4) (Elbadrawy & Sello, 2016); (5) (Martin & Rose, 2014); (6) (Ferreira et al., 2012). (7) (Ferguson & Harris, 1998);*Value correspondent to non-starch polysaccharides calculated from neutral monosaccharide x 0.89 + uronic acid x 0.91 content in dry potato skins. ** Values calculated from nutrient content(g)/(100g potato skin – water content(g))x100; (8) (Isaacson et al., 2009)

Characterization of biomass

- **Acid hydrolysis of polysaccharides**

The hydrolysis of the polysaccharides with concomitant release of the corresponding relied on an acid hydrolysis method following a protocol already described (Silva, 2015). In brief, 0.1mL of 76% Sulfuric Acid were added to a test tube with 0.01g of biomass, left for 1h at 30°C. Next, the pH of the solution was elevated to about 2 (sulfuric acid is diluted to about 3%) with the addition of 2.8mL of distilled water and left for 1h at 121°C. The reaction was stopped by putting the test tubes on cold water. Once ambient temperature was reached, the solution was filtered and the filtrate was kept in a new test tube in a cold chamber to avoid further degradation.

- **Quantification of polysaccharides by the phenol-sulphuric acid colorimetric method**

This method followed a previously described protocol (Silva, 2015). After the acid hydrolysis aforementioned, 1.5mL of 96% Sulfuric Acid and 0.3mL of 5% Phenol were added to 0.5mL of the resulting filtrate. This solution was kept for 5 minutes at 90°C. After cooled in a water bath, the absorbance at 490 nm was read in a polystyrene non-pyrogenic microplate (Corning PrimariaTM Multiwell Cell Culture Plate; 96-well flat bottom with low evaporation lid) on a Tecan Infinite200 Spectrophotometer (Tecan, Norway) through the Tecan i-Control Software (v. 1.4.50).

- **Quantification of polysaccharides by HPLC – High-Performance Liquid Chromatography**

The sugar quantifications on the various sources were done by high performance liquid chromatography (HPLC) using an Alliance 2695 Waters chromatographer (Waters Chromatography, Milford, MA), connected to a LKB 2142 Differential Refractometer (Bromma, Sweden) detector. Data acquisition and processing was accomplished with the Empower 2 software (Waters Chromatography). Chromatographic separation was undertaken using an Aminex HPX-87H column (300 x 7.8 mm), 9 µm particle size (Bio-Rad, Hercules, California) and set at 60°C. Elution through the column was carried out isocratically at a flow rate of 0.5 mL/min, with 0.005 N of Sulfuric Acid (H_2SO_4) with an injected volume of 90 µL. The retention times (RT) of the compounds were compared with glucose (RT=10.254min), fructose (RT=11.029min), ribitol (RT=11.974min) and sorbitol (RT=11.538min) standards for identification, and the peak area was used for quantification.

- **ATR FT-IR – Fourier Transform Infrared Spectroscopy**

Spectra from all sources were obtained on a Bruker IFS66/S FTIR Spectrometer (Bruker Daltonics, MA, USA) equipped with a single reflection ATR cell (DuraDisk with diamond crystal) to qualitatively follow compositional alterations of the biological material before and after pre-treatment with Soxhlet extraction and/or enzymatic hydrolysis, specially the prevalence of the peaks assigned to the polyester. Table 1.2 below contains the peak assignments that are most relevant for the ATR FT-IR spectra analysis.

Table 1.2 - ATR FT-IR absorbance peaks in cm-1 and related band assignments for cutin and suberin polymers. In bold are the dominating peaks in suberin extracted from cork (Ferreira et al., 2012) and cutin extracted from with mature tomato fruit cuticles (Benítez, Matas, & Heredia, 2004), presenting slight deviations.

Cutin	Suberin	Band assignment
3320	3330	O-H stretching vibration of carboxylic acids and alcohol groups
2924	**2920**	Asymmetric and symmetric C-H stretching vibrations
2852	**2851**	
1730	**1735**	C–O stretching vibration of the carbonyl group of the ester bond
1463	1463	$\delta(CH_2)$ scissoring
	1245	Symmetric C–O stretching
	1164	Asymmetric C–O stretching
1169	1130	va(C- O-C)
1100		v(C–H)
1055	1050	v(C–O)
724	**722**	C–H bend

- **GC-MS**

The GC-MS protocol was optimized at ITQB-NOVA and the identification of the compounds was based on the equipment spectral library Wiley-Nist and previously published data (Heinämäki et al., 2017)(Lopes et al., 2000)(Cordeiro, Belgacem, Silvestre, Pascoal Neto, & Gandini, 1998)(Pinto et al., 2009) focusing their EI-MS fragmentation patterns and/or retention times. Graphic construction was based on the percentage of a given compound in the precise mass that was weighted for sampling, following the equation below

$$(1)\ \frac{\boldsymbol{\mu g\ of\ compound}}{\boldsymbol{ug\ of\ sample}} \times \mathbf{100}$$

Suberin from 2-hour extraction of cork, suberin from white potato skin and cutin from tomato skin were analyzed in triplicate and suberin from 1-hour extraction of cork, due to lack of biomass and the destructive nature of GC-MS, was analyzed in duplicate.

Suberin

An Agilent (7820A) gas chromatograph equipped with an Agilent (5977B) mass spectrometer was used. The GC-MS was first calibrated with pure reference compounds (representative of the major classes of compounds present in suberin) relative to n-hexadecane (internal standard). Samples were submitted to alkaline hydrolysis prior to the methylation and silylation, to release hydrolysable monomeric constituents. Briefly, suberin samples were treated with a solution of 0.5 M NaOH in methanol/water (1:1, v/v) at 95 ºC, during 4 hours. The mixture was cooled to room temperature, acidified to pH 3–3.5 with 1M HCl, and extracted three times by dichloromethane/water partition. The combined organic extracts were dried in a rotary evaporator, then trimethylsilylated as mentioned above, and analysed by GC-MS using a HP-5MS with the following ramp temperature: 80^{o}C, 4^{o}C/min until 310^{o}C, 310^{o}C for 15 min.

Cutin

An Agilent (7820A) gas chromatograph equipped with an Agilent (5977B) mass spectrometer was used. The GC-MS was first calibrated with pure reference compounds (representative of the major classes of compounds present in cutin) relative to heptadecane and omega-pentadecalactone (internal standards). Samples were submitted to acidic hydrolysis prior to the silylation, to release hydrolysable monomeric constituents. Briefly, suberin samples were treated with a solution of 5% (w/v) in methanol at 85 ºC, during 3 hours. The mixture was cooled to room temperature, extracted two times by dichloromethane/NaCl 2.5% (w/v) partition and organic phase washed with Tris NaCl (Tris 100 mM NaCl 0.09%). The combined organic extracts were dried in a rotary evaporator, then trimethylsilylated as mentioned above, and analysed by GC-MS using a HP-5MS with a the following ramp temperature: 50ºC/min 1 min, 25ºC/min until 150ºC, 150ºC during 2 min, 10ºC/min until 320ºC and 320ºC for 6 min.

Biopolymer isolation through selective depolymerization

As mentioned previously, cholinium hexanoate specifically cleaves acylglycerol ester bonds in suberin, while leaving linear aliphatic bonds intact. The experiment followed a method previously described (Garcia et al., 2010) with slight modifications. Briefly, the biomass was mixed with the previously dried ionic liquid in a proportion of 1:9 (w/w) and the mixture was left in a heater for a period of 30 minutes, 1 hour or 2 hours, under 100^{o}C without agitation. The

subsequent steps to isolate the biopolymer are described in Figure 1.2 and represent an upgraded version (still under development) of the previous published polyester recovery method.

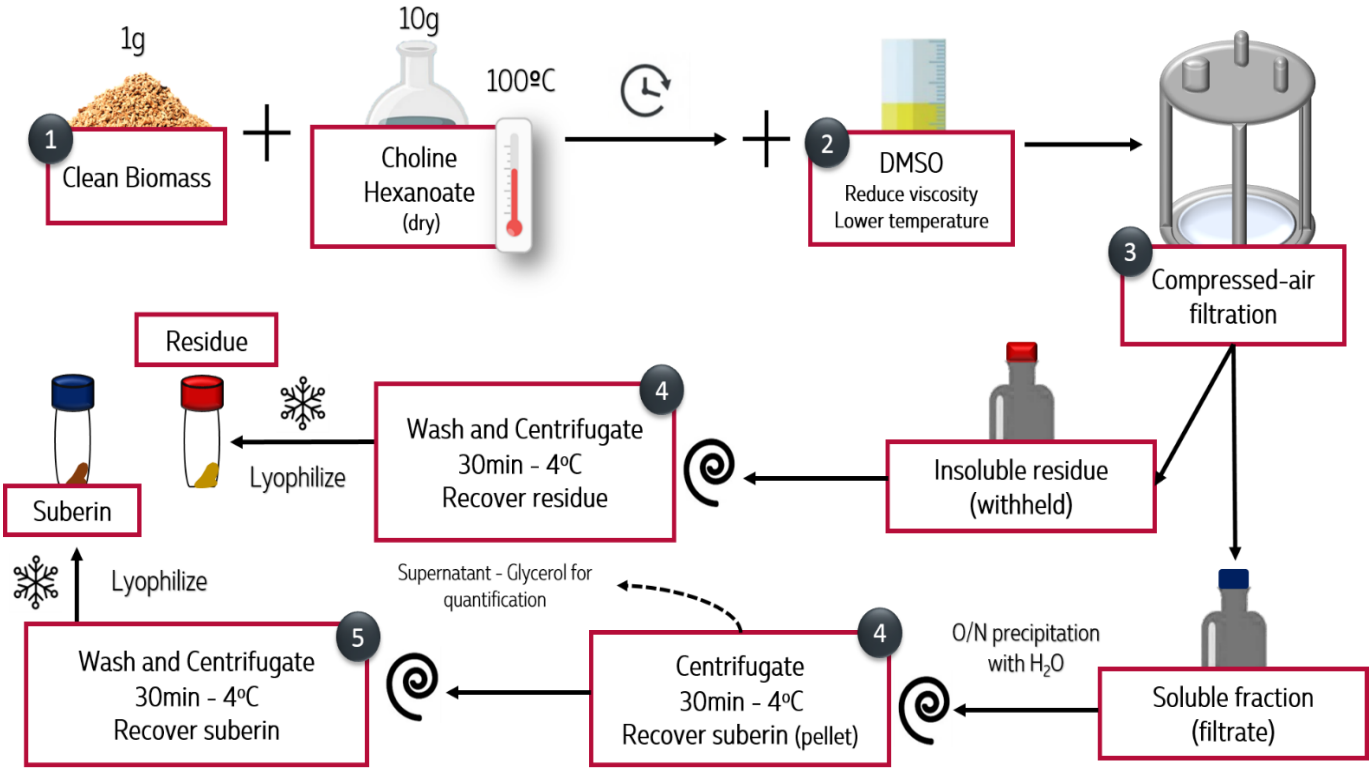

Figure 1.2 – Schematic representation of cholinium Hexanoate-mediated extraction of biopolymers.

1.3 Results and Discussion

Soxhlet extractions

By way of example, only the mass balance results for tomato skin and white potato skin are shown.

Table 1.3 – Mass balance for Soxhlet extraction of tomato peels and white potato skin

	Weight before Soxhlet (g)	**Weight after Soxhlet (g)**
Tomato peels (g)	4.58	3.95
Biomass loss (%)	**-**	**13.75**
White potato peels (g)	1.86	0.93
Biomass loss (%)		**50.00**

Results show that about 14% of the dried tomato peels and around 50% of the dried white potato peels represent soluble components that could be extracted. This could mean that

roughly 86% of tomato peels and 50% of white potato peels potentially have cutin and suberin, respectively.

Enzymatic digestion

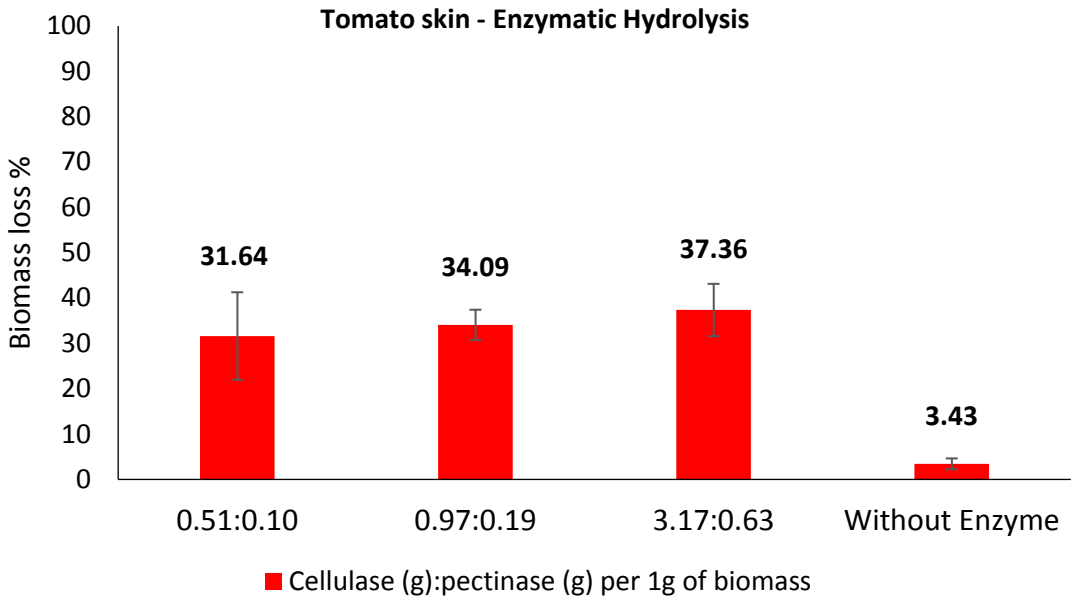

Figure 1.3 - Results of enzymatic digestion of tomato skin using three different enzymatic ratios.

Results for the enzymatic digestion of tomato skin presented in Figure 1.3 suggest that the treatment was very efficient given that the loss of biomass was ≈11 times higher in enzyme tests compared to the control tests. The three enzymatic ratios led to similar losses of biomass,

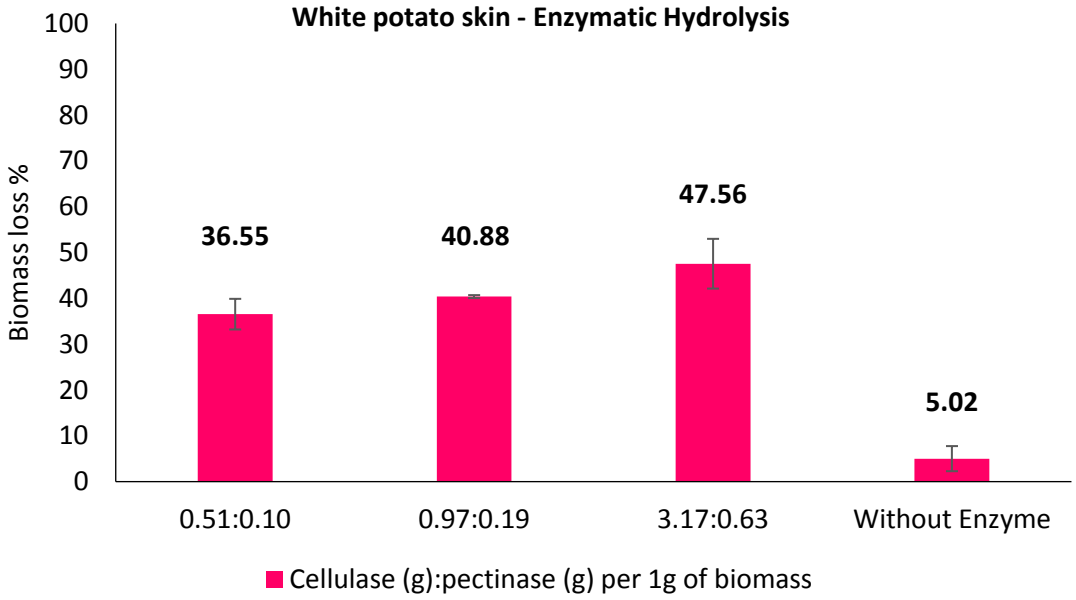

Figure 1.4 – Results of enzymatic digestion of white potato skin using three different enzymatic ratios.

The enzymatic digestion under the conditions already described was efficient in the case of white potato peels (Figure 1.4) with a loss of biomass ≈9 times higher than the control test without enzyme. Despite higher loss of biomass for the enzymatic ratio of 3.17:0.63, given its higher standard deviation the results suggest that the more reliable and less costly treatment is the enzymatic hydrolysis with the ratio of 0.97:0.19.

The enzymatic digestion was not applied to refined cork given its already low content of polysaccharides (Table 1.1).

Characterization of biomass

- **Phenol-Sulphuric acid colorimetric method**

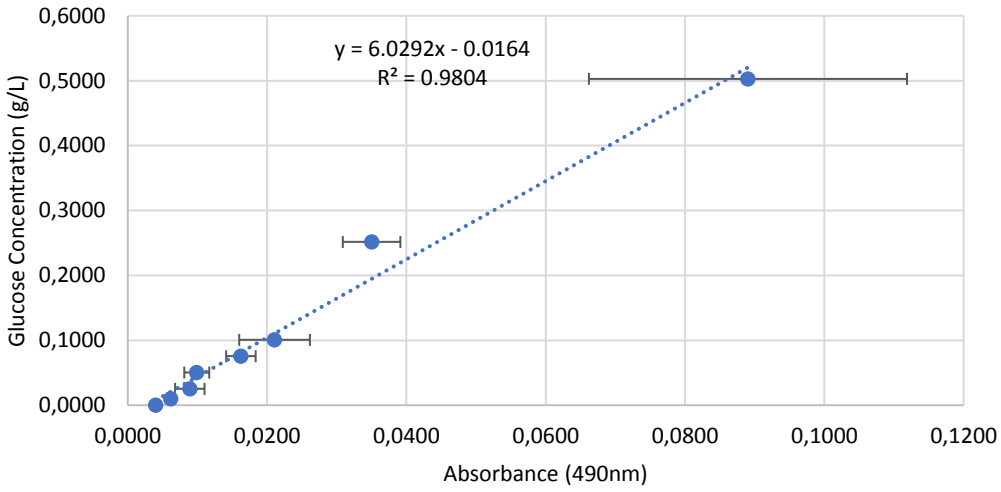

Figure 1.5 - Calibration curve for glucose concentration (g/L).

The results for this method were based on calculations using the equation for the calibration curve of glucose, shown in Figure 1.5. This calibration curve was initially made with glucose concentrations in the range of 0 to 1g/L, but R values started to drop significantly above 0.5g/L. To calculate the sugar concentration in each sample (g/L), after the phenol-sulfuric acid method and absorbance reading, the equation (2) was applied

$$(2)\ \boldsymbol{y = (6.0292x - 0.0164) \times dilution\ factor}$$

Because the reaction volume of the acid hydrolysis was 2.9mL (0.100mL Sulfuric acid + 2.8mL Distilled Water), we can obtain the polysaccharide mass (g) in the sample with the following reason (3) and (4):

$$(3)\ \boldsymbol{[Polysaccharide]g/L\ - 1000mL}$$

$$(4)\ \boldsymbol{Polyssacharide\ g\ - 2.9mL}$$

The polysaccharide content is then obtained by dividing the polysaccharide mass (g) calculated for each sample by the biomass weighted for each tube (5):

$$(5)\ \ \boldsymbol{Polysaccharide\ content}\ \% = \frac{\boldsymbol{Polysaccharide(g)}}{\boldsymbol{Sample(g)}}$$

Table 1.4 – Polysaccharide content in treated and untreated tomato skin calculated through the phenol-sulphuric acid colorimetric method.

Tomato Skin							
Enzymatic ratio	**Sample (g)**	**Abs (490nm)**	**Dilution**	**[Polysaccharide] g/L**	**Polysaccharide (g)**	**Polysaccharide (%)**	**STD.DV**
0.51:0.10	0.0010	0.0584	1	0.3357	0.0010	97.3545	25.84
0.51:0.10	0.0010	0.0793	1	0.4617	0.0013	133.8975	
Source	0.0010	0.0590	10	3.3932	0.0098	984.0361	44.58
Source	0.0010	0.0554	10	3.1762	0.0092	921.0913	

The results obtained for tomato skin (Table 1.4) reveal this method is not reliable nor reproducible, particularly when the mass of sample used is below 0.005g and a dilution factor is applied. Because of these results, HPLC was performed on tomato skin and white potato skin to obtain more precise results.

- **HPLC**

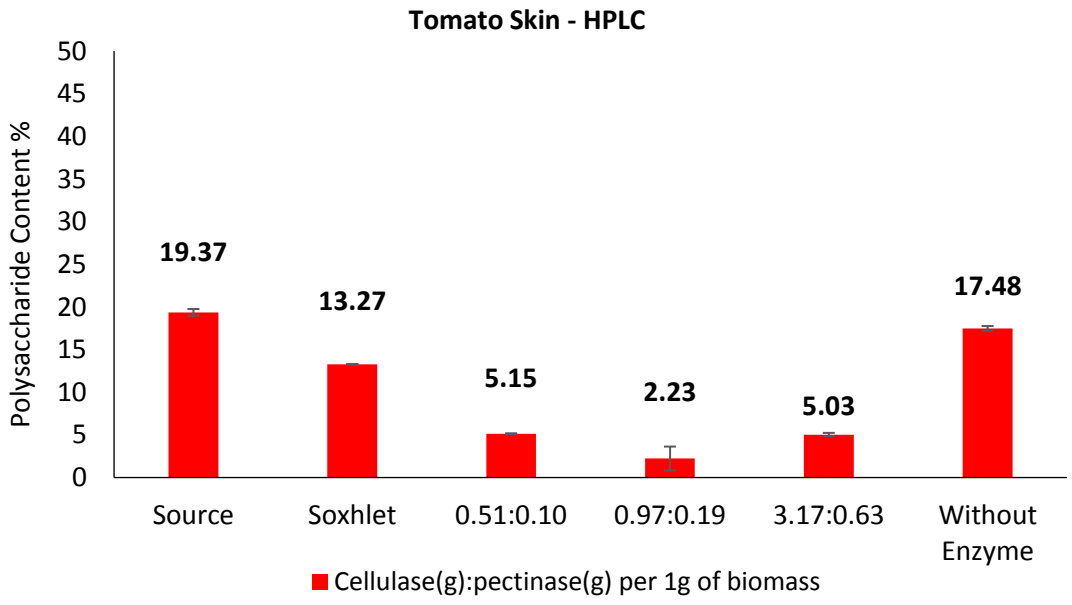

Figure 1.6 – HPLC results for polysaccharide content in tomato skin.

The results in Figure 1.6 are consistent with the mass balance calculations for enzymatic hydrolysis of tomato skin, given the lower polysaccharide content of the treated material when compared to the control test, the untreated source and the post-Soxhlet source. The best enzymatic treatment appears to be 0.97g of cellulase:0.19 g of pectinase per 1g of biomass. In this case, the calculated content in the source – 19.37% – differs from the one found in literature (≈ 30%) (Elbadrawy & Sello, 2016).

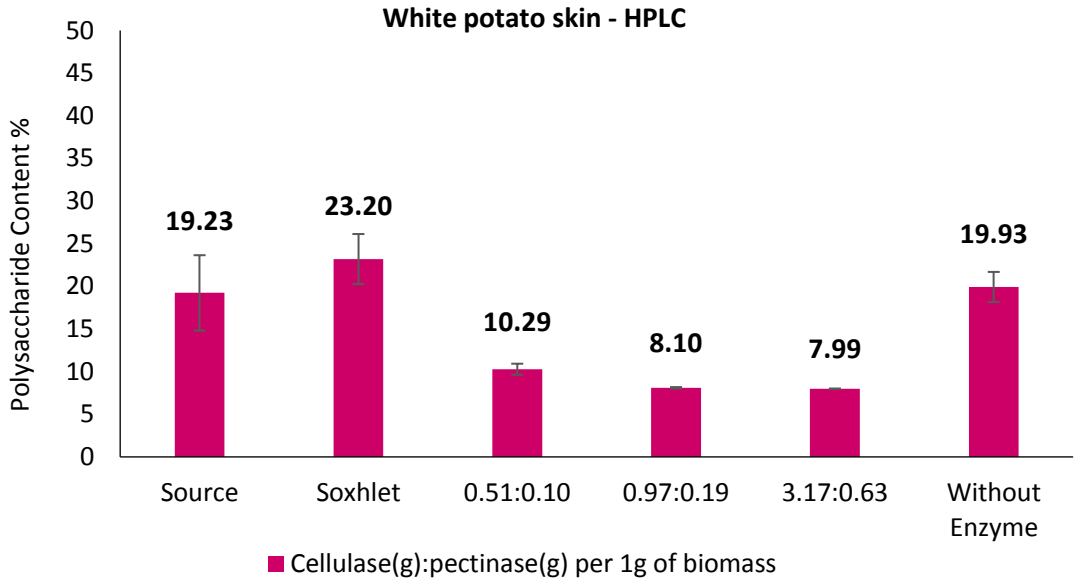

Figure 1.7 - HPLC results for polysaccharide content in white potato skin.

The results in Figure 1.7 are consistent with the mass balance calculations for enzymatic hydrolysis of white potato skin. HPLC data suggest a lower polysaccharide content in the material that lost more biomass after the enzymatic hydrolysis and once again, the best enzymatic treatment appears to be 0.97g of cellulase:0.19 g of pectinase per 1g of biomass. Data is also consistent with the fact that post-Soxhlet/pre-enzymatic hydrolysis and the control test both show higher polysaccharide content and are similar to each other. The experimentally calculated polysaccharide content in the source – 19.23% – differs from one found in literature (≈30%) (See Table 1.1).

- **ATR FT-IR**

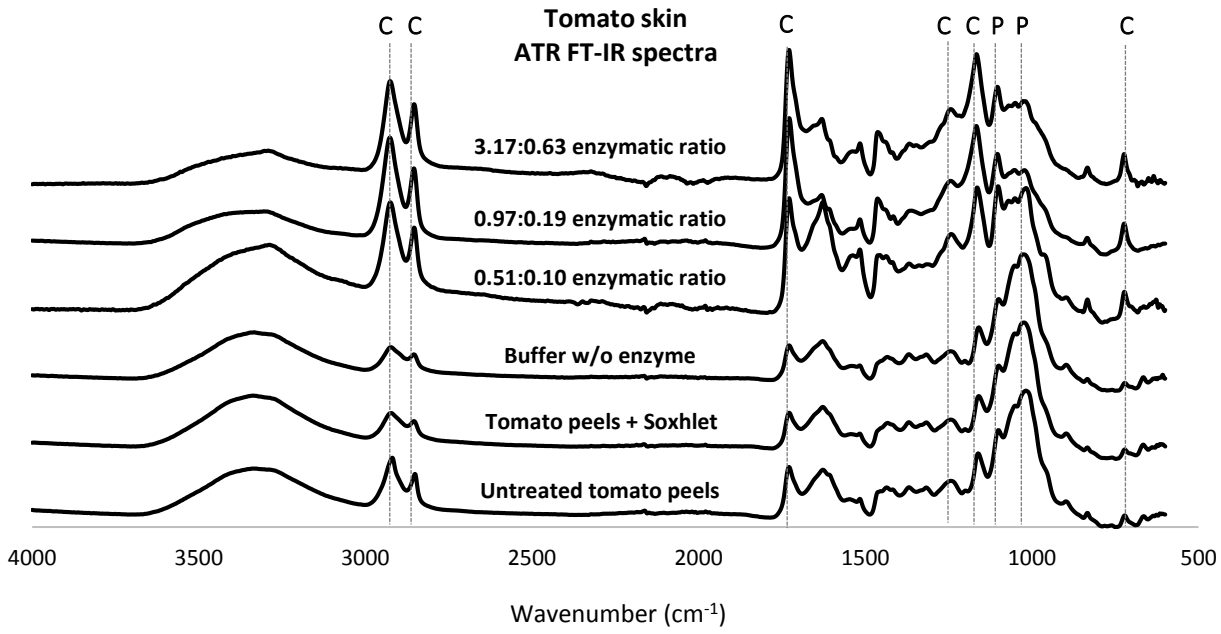

Figure 1.8 - ATR FT-IR spectra of the pre- and post-treated tomato peels. (C) = Suberin; (P) = Polysaccharides

ATR FT-IR spectra of tomato skins (Figure 1.8) submitted to either only Soxhlet extractions or enzymatic hydrolysis compared to untreated skins suggest a visible decrease in polysaccharide related peaks (1097 and 1012cm^{-1}) and, accordingly, a relative increase in main polyester related peaks (2931, 2858 and 1724 cm^{-1}). These results further corroborate this choice of treatment before cholinium hexanoate extraction.

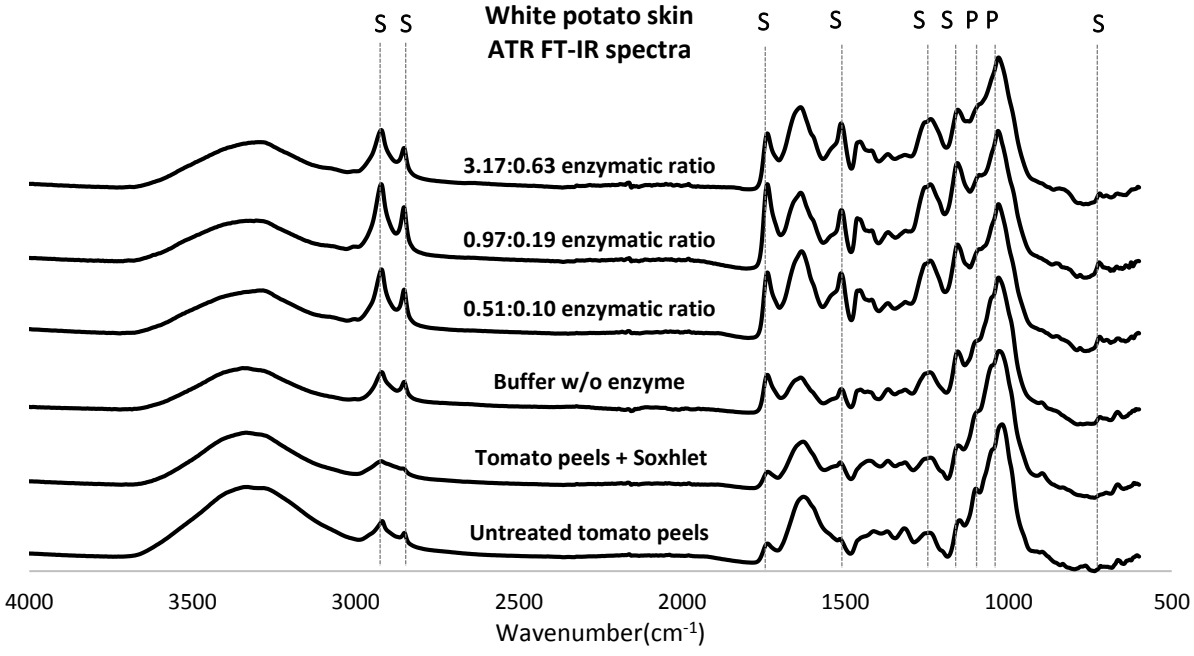

Figure 1.9 - ATR FT-IR spectra of the pre- and post-treatments of white potato peels. (S) = Suberin; (P) = Polysaccharides

ATR FT-IR spectra of white potato skin submitted to the various conditions described (Figure 1.9) also suggest a decrease in polysaccharides in the enzymatically treated biomass (1130-975cm^{-1}) and, accordingly, a relative increase in polyester (2910 and 2844cm^{-1}). The compositional modification of the peels submitted to enzymatic hydrolysis was more evident when using the cellulase:pectinase ratio of 0.97:0.19. The peak at 3271cm^{-1} appears smaller (O=H stretching) and C=O ester group stretching at 1749cm^{-1} appears more evident, which also further corroborates HPLC and mass balance results.

Biopolymer yield

While the purpose was to obtain biopolymers from both treated and untreated biomasses, this protocol was only implemented on the extraction of suberin from post-Soxhlet cork, suberin from untreated white potato skin and cutin from untreated and enzymatically digested tomato skin, which was due to the excessive time the ionic liquid takes to dry. Also, biomass loss (%) was calculated using the equation (6):

$$(6)\ \frac{(\boldsymbol{Initial\ Biomass} - (\boldsymbol{Biopolymer\ mass} + \boldsymbol{insoluble\ residue\ mass}))}{\boldsymbol{Initial\ biomass}} \times \mathbf{100}$$

Table 1.5 – Polymer yields for the 1st extraction of suberin from cork.

Suberin from refined cork – 1st extraction

Extraction time (h)	Initial biomass (g)	Biopolymer mass (g)	Extraction yield (%)
0.5	0.90	0.02	**2.22**
1.0	0.90	0.04	**4.44**
2.0	0.93	0.18	**19.35**

The extractions of suberin from cork (Table 1.5) served as the control extraction, given the known results from previous published experiments (Ferreira et al., 2012). Some leakage of DMSO + Cork + IL occurred during the 1-hour extraction (**) and residues from all extractions got lost, hence, a second extraction was performed however using less initial biomass and, consequentially, less ionic liquid.

Table 1.6 - Polymer yields for the 2nd extraction of suberin from cork.

Suberin from refined cork – 2nd extraction

Extraction time (h)	Initial biomass (g)	Biopolymer mass (g)	Extraction yield (%)
0.5	0.20	-	**-**
1.0	0.20	0.02	**10.00**
2.0	0.20	0.04	**20.00**

The 2nd set of extractions of suberin from cork (Table 1.6) resulted in a vestigial amount of biopolymer for the 30-minute test, possibly because the yield is not sufficiently high to allow recovery from such a small amount of initial biomass, meaning it is only possible to estimate values with more biomass. The 2-hour extraction resulted in the same yield as the previous set of extractions and, as expected, the 1-hour extraction resulted in a higher extraction yield than before.

Table 1.7 – Insoluble residue yields for the 2nd extraction of suberin from post-Soxhlet cork extractions.

Suberin from refined cork – Insoluble residue

Extraction time (h)	Initial biomass (g)	Residue mass (g)	Biomass Loss (%)
0.5	0.20	-	**-**
1.0	0.20	0.15	**15.00**
2.0	0.20	0.11	**25.00**

Residue calculations (Table 1.7) are valuable because, in theory, obtained biopolymer mass summed with the insoluble residue mass should be the closest to the initial biomass. However, this proposition was not confirmed with neither extraction (15% and 25% for 1-hour extraction and 2-hour extraction, respectively) which might be due to biomass loss during filtration or washing steps. After evidence that the 2-hour extraction would more likely result in higher biopolymer recovery yields, the next extractions followed the same procedure.

Table 1.8 – Mass balance for extractions of suberin from white potato skin and cutin from tomato skin.

	Initial biomass (g)	Polyester (g)	Extraction yield (%)	Insoluble residue (g)	Biomass loss (%)
Suberin from white potato skin	2.00	0.07	3.50	1.10	**41.50**
Cutin from tomato skin (untreated)	2.11	0.12	5.69	1.45	**25.59**
Cutin from tomato skin (treated)	1.59	0.01	0.63	1.38	**12.58**

While the biopolymer yield for white potato peels extraction (Table 1.8) was much lower in comparison to cork, the mass balance results for insoluble residue + biopolymer suggests an even higher biomass loss during the process (41.5%)

As for tomato peels, mass balance calculations reveal that the extraction from the enzymatic hydrolyzed material resulted in a polymer yield around 9 times lower than the extraction of untreated material. However, biomass loss decreased practically half (from 25.6% to 12.6%, respectively).

Biopolymer characterization

- **ATR FT-IR**

The spectra from the extracted biopolymers and insoluble residues were obtained on the same spectrometer used for the initial biomass characterization. The main difference between readings of the initial biomass, the residue and the polymer, is the nonexistence of biological replicas of the latter due to the small amounts available, while other samples had 2 biological replicas, each read 3 times. Nonetheless, 3 technical replicas were made for each biopolymer.

For the analysis of the ATR FT-IR spectra, cutin and suberin related peaks have must be taken in consideration. These have already been described (Benítez et al., 2004)(Cordeiro et al., 1998) and are described in Table 1.2.

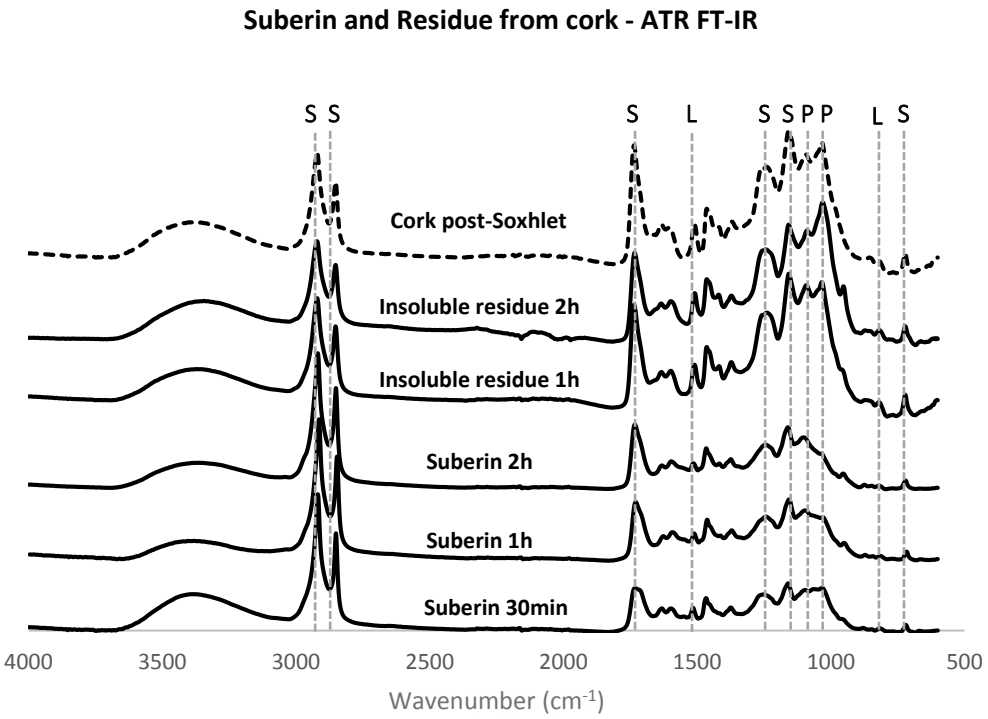

Figure 1.10 - ATR FT-IR spectra of cork, extracted suberin and insoluble residue. (S) Suberin; (P) Polysaccharides; (L) Lignin

The ATR FT-IR spectra of suberin extracted from cork (Figure 1.10) reveal, though with some deviation from literature, the presence of the major suberin related peaks (2891, 2827 and 1724cm^{-1}), with no relevant differences between the suberin extracted for 30 minutes, 1 hour and 2 hours. The presence of peaks related to suberin is still detected in the insoluble residue. The suberin samples show a relative decrease in the polysaccharide related peaks (1088 and 1031cm^{-1}), compared to the insoluble residue and cork. Lignin related peaks (1512 and

810cm^{-1}), associated with the presence of cellulose, also show relative decrease in suberin samples. No insoluble residue from the 30-minute extraction could be recovered.

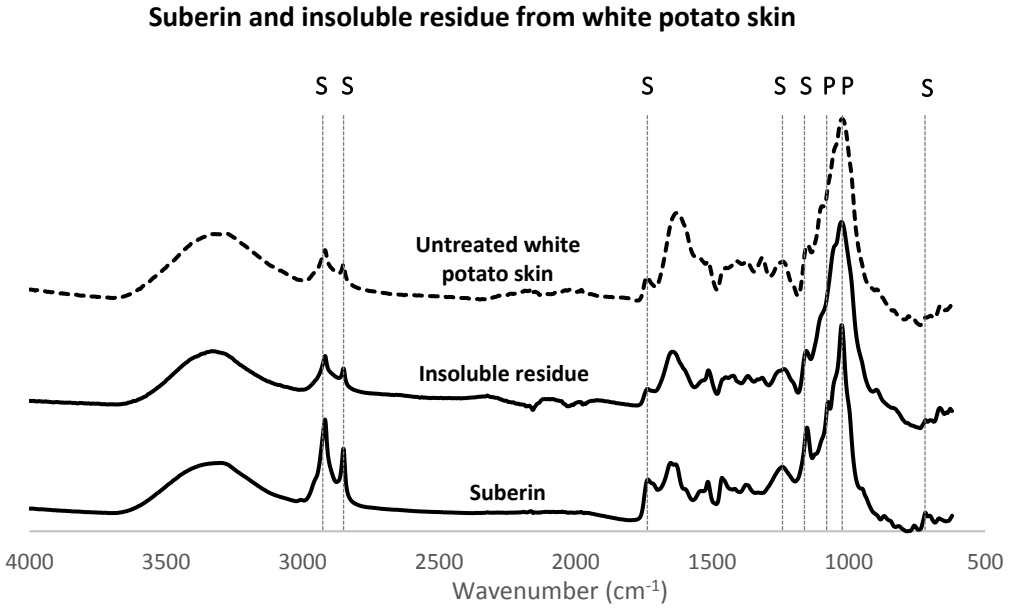

Figure 1.11 - ATR FT-IR spectra of white potato skin, extracted suberin and insoluble residue. (S) Suberin; (P) Polysaccharides.

The ATR FT-IR spectra in Figure 1.11 is very similar to the previous one. Again, suggesting a high contribution of suberin related peaks in the potato suberin sample (2908, 2842 and 1720 cm^{-1}), with a slight deviation from peaks described in literature (Table 1.2). However, the relative decrease in polysaccharide related peaks is much less evident. The peaks assigned to suberin are still present in the insoluble residue and in the source although with lower absorbance.

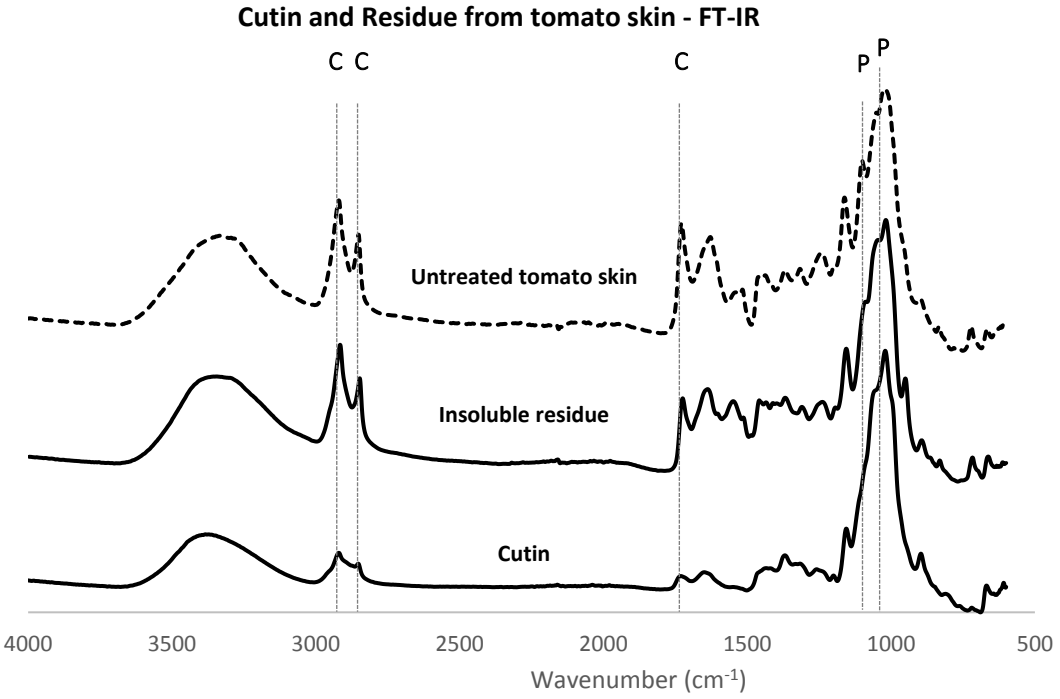

Figure 1.12 - ATR FT-IR spectra of tomato skin, extracted cutin and insoluble residue comparison. (C) Cutin; (P) Polysaccharides.

Unlike the previous spectral analysis, the ATR FT-IR spectra of cutin from untreated tomato skin (Figure 1.12) shows very low polymer assigned peaks in the cutin (2921, 2844 and 1718cm^{-1}), contrary to their high contribution in the insoluble residue and in the source. The polysaccharide related peaks are pratically unchanged in all samples (1093 and 1026 cm^{-1}).

- **GC-MS**

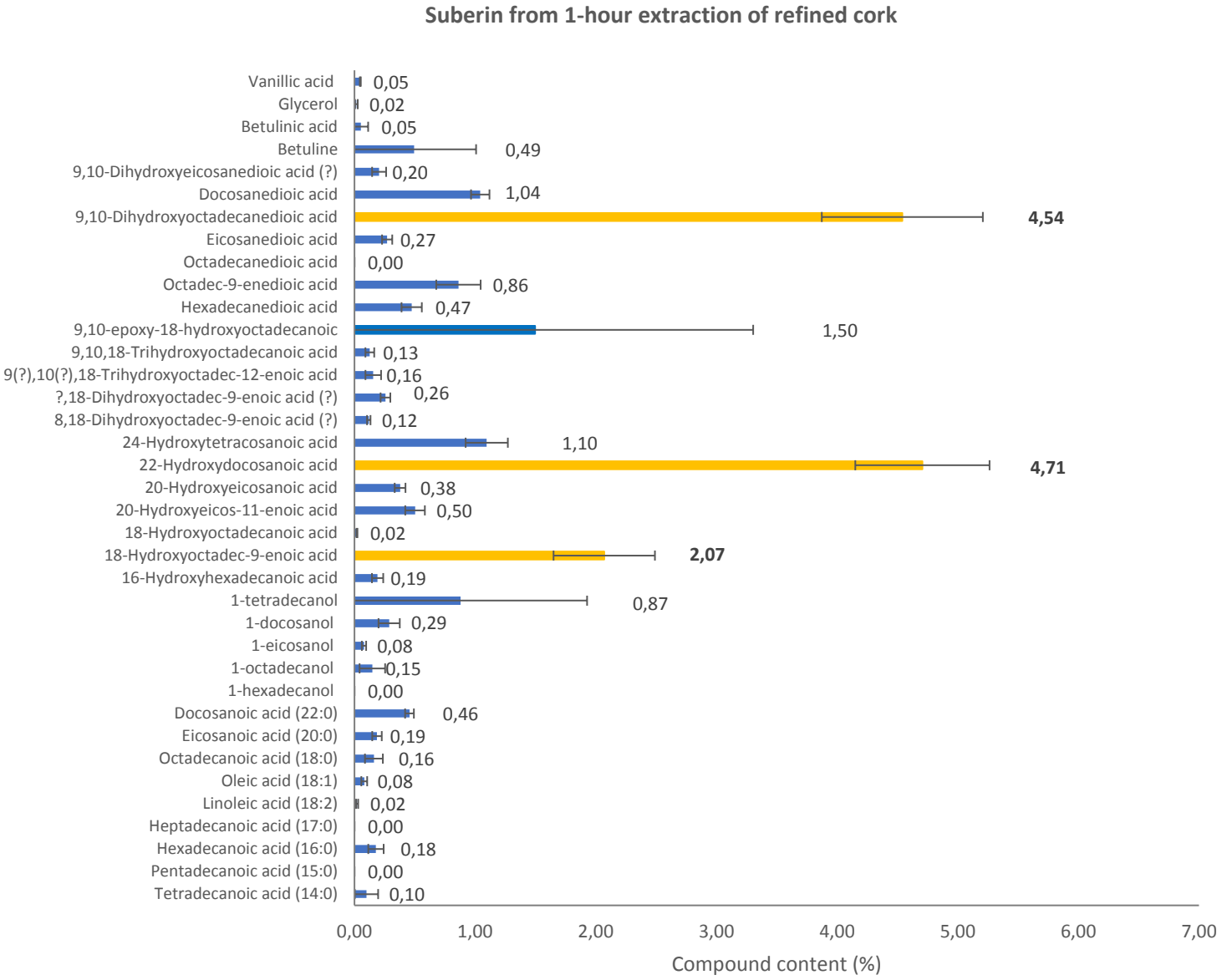

Figure 1.13 – GC-MS calculated monomeric content of suberin extracted from cork for 1 hour.

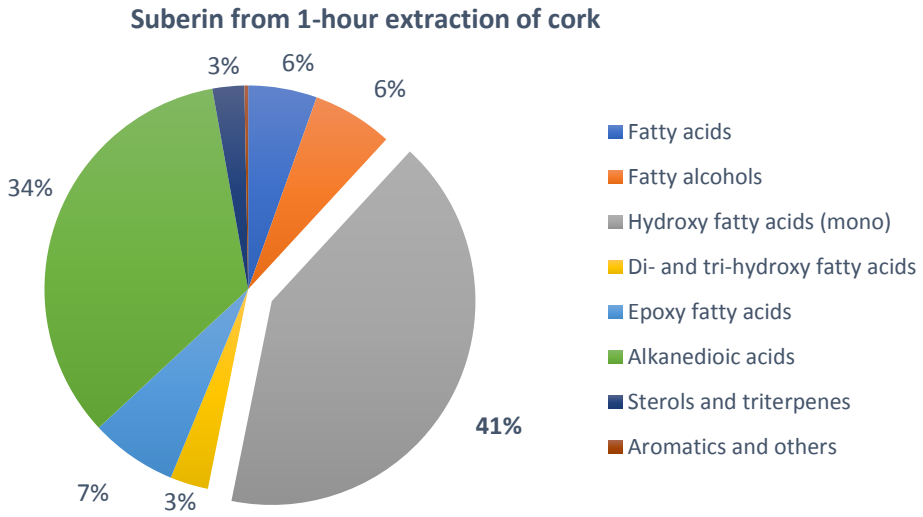

Figure 1.14 - Families of compounds present in suberin from 1-hour extraction of cork; percentages are related only to the total of components identified through GC-MS (21.70%).

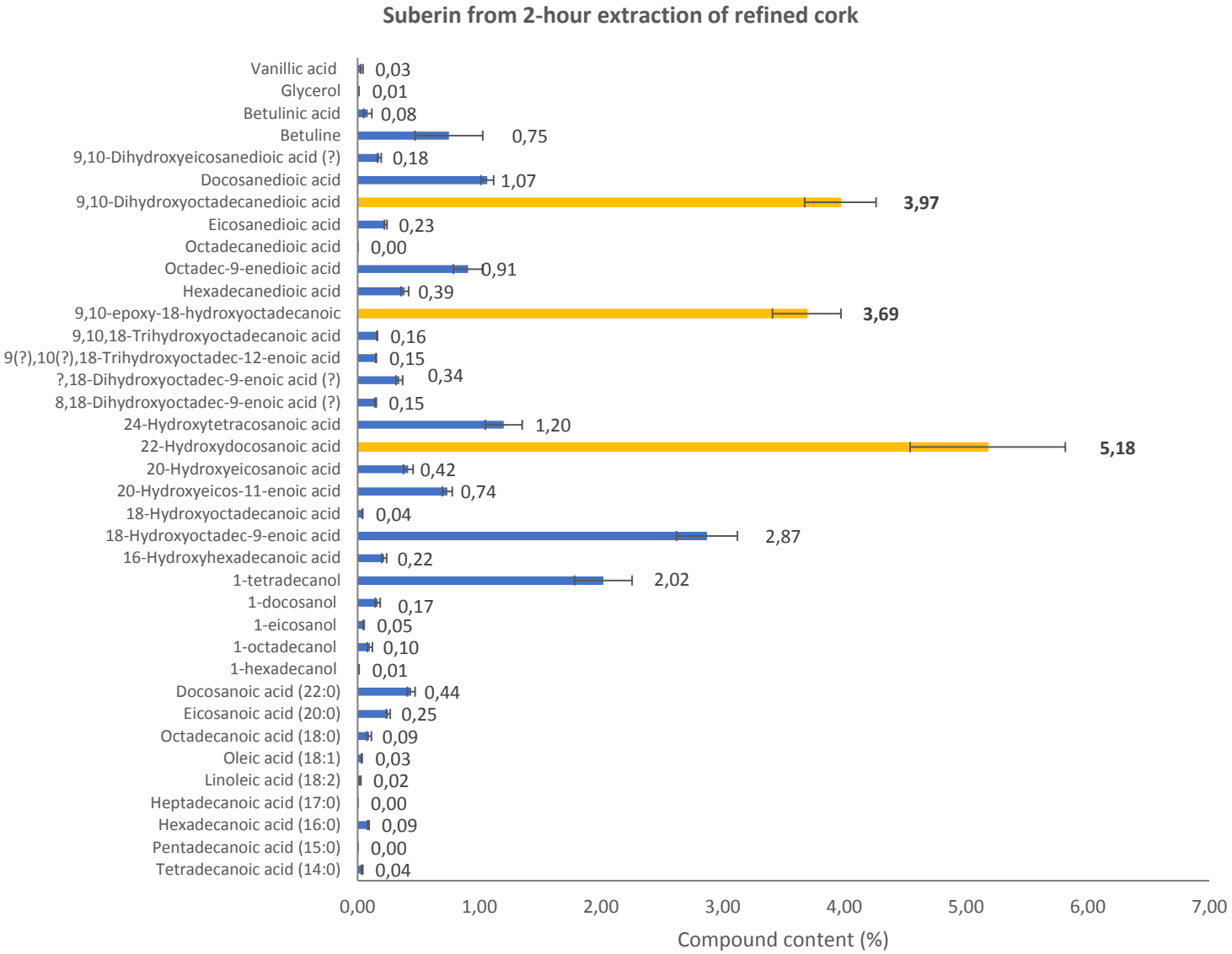

Figure 1.15 - GC-MS calculated monomeric content of suberin extracted from cork for 2 hours.

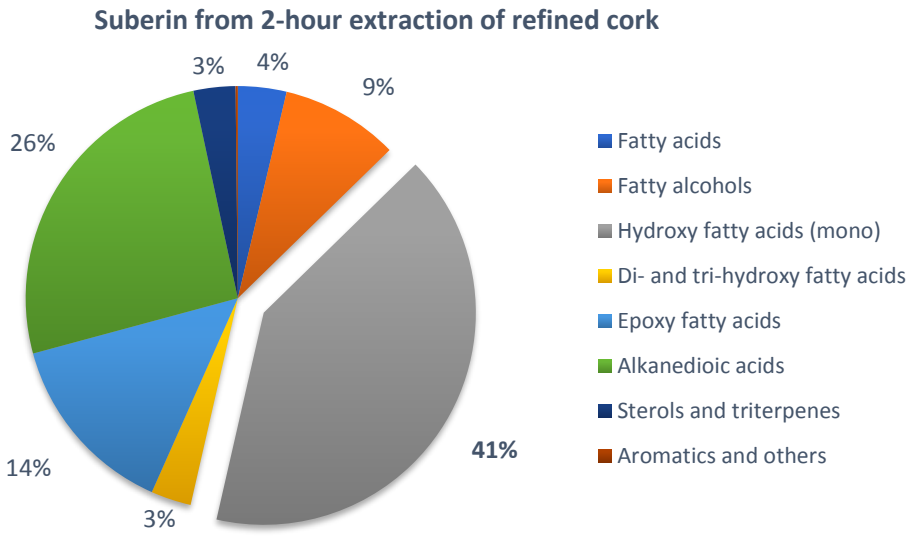

Figure 1.16 - Families of compounds present in suberin from 2-hour extraction of cork; percentages are related only to the total of components identified through GC-MS (26.08%).

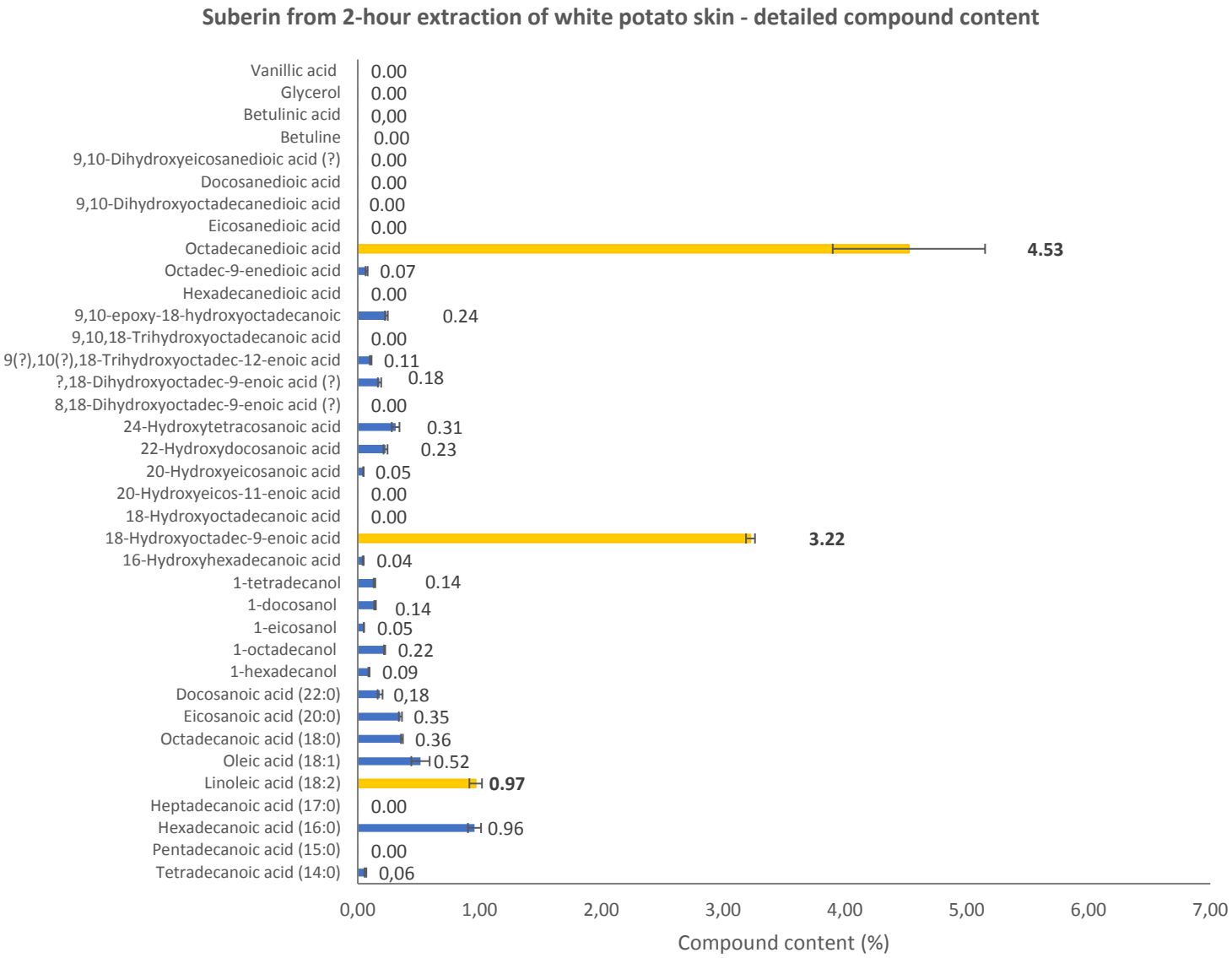

Figure 1.17 - GC-MS calculated monomeric content of suberin extracted from white potato skin for 2 hours.

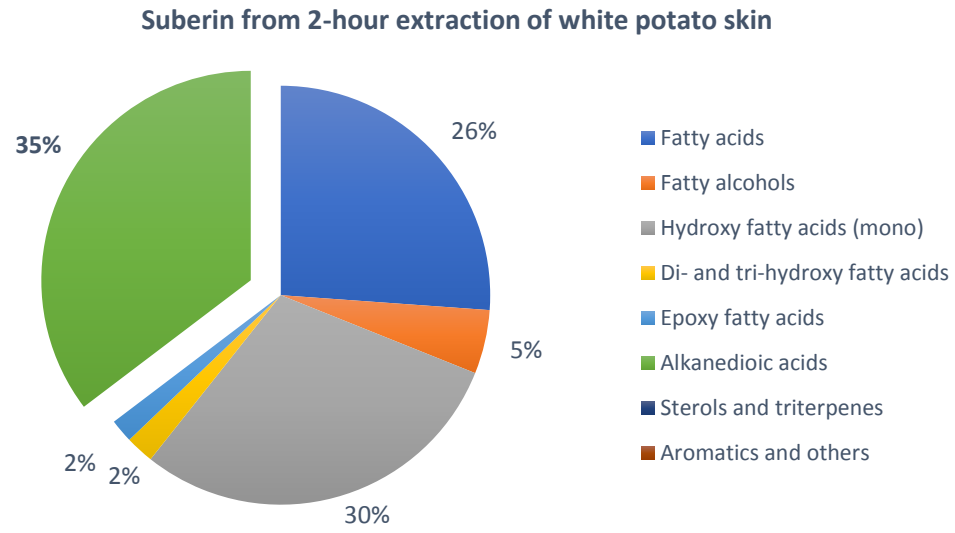

Figure 1.18 - Families of compounds present in suberin from 2-hour extraction of white potato skin; percentages are related only to the total of components identified through GC-MS (13,02%).

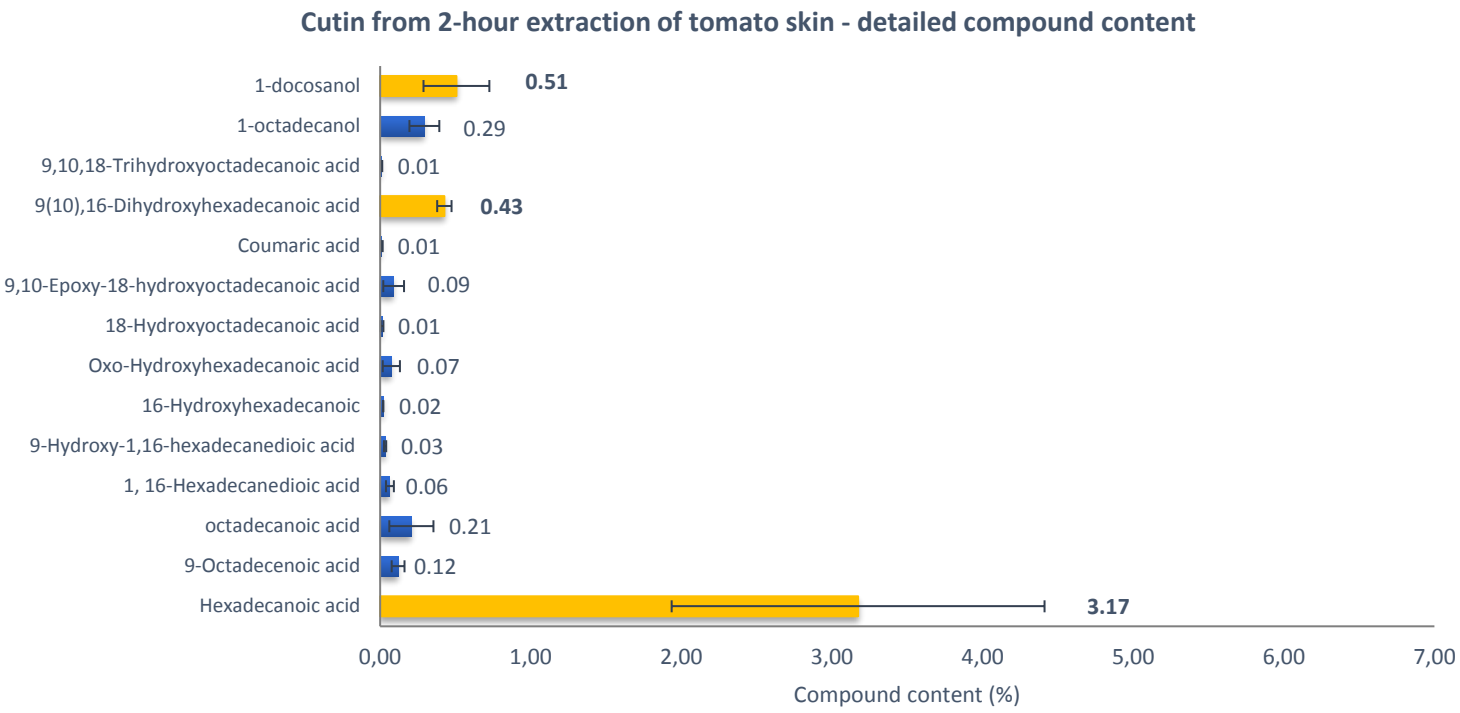

Figure 1.19 - GC-MS calculated monomeric content of cutin extracted from tomato skin for 2 hours.

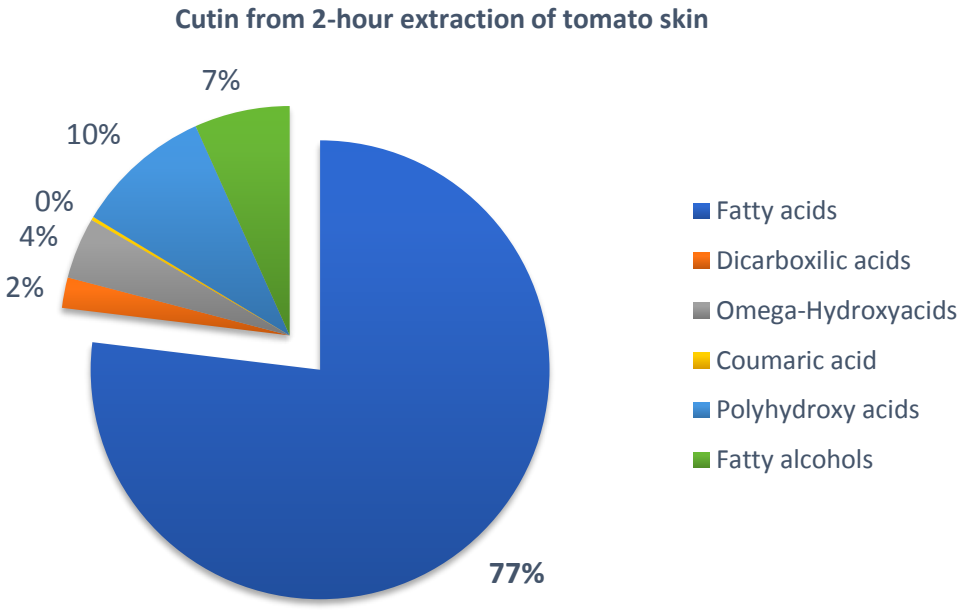

Figure 1.20 - Families of compounds present in cutin from 2-hour extraction of tomato skin; percentages are related only to the total of components identified through GC-MS (4.55%).

Suberin from 1-hour extraction of cork

GC-MS of suberin extracted from granulated cork for 1-hour allowed identification a total of 21.70% of compounds (Table 1.9 in Appendix), which means 78.30% is left unidentified. Nonetheless, of the total compounds identified, the majoraty belongs to the family of hydroxy fatty acids, with **22-Hydroxydocosanoic acid** as major contributor, and the family of alkanedioic acids mainly represented by **9,10-dihydroxyoctadecanedioic acid**.

Suberin from 2-hour extraction of cork

GC-MS of suberin extracted from granulated cork for 2-hour allowed identification a total of 26.08% of compounds, meaning 73.92% of compounds were not identified. Like the previous polyester, the hydroxy fatty acid **22-Hydroxydocosanoic acid** and the alkanedoic acid **9,10-Dihydroxyoctadecanedioic acid** are the major contributors.

Suberin from 2-hour extraction of white potato skin

For this polyester, GC-MS permitted the identification of only 13.02% of compounds, leaving 86.98% of compounds unidentified. While still mainly represented by alkanedioic acids and hydroxy fatty acids, like the two previous suberin samples, the main representative of first is **octadecanedioic acid**, while **18-Hydroxyoctadec-9-enoic acid** is the major representative of hydroxy fatty acids.

Cutin from 2-hour extraction of tomato skin

Highly contrasting with the three previous biopolymers, GC-MS of cutin only allowed identification of 4.55% of compounds, leaving 95.45% of compounds unidentified. Nevertheless, from the total of compounds acknowledged, a great majoraty of them are fatty acids, namely **Hexadecanoic acid**. The second most abundant compound identified is **1-docosanol** from the family of fatty alcohols and the third most abundant compound was **10,16-dihydroxyhexadecanoic acid** from the family of polyhydroxy acids.

1.4 Discussion

The enzymatic digestion of the biopolyester sources led to mass losses of about 7-9x for white potato peels, and about 10-12x higher for tomato peels, along with consistent HPLC results for quantification of polysaccharides, supporting a successful removal of some polysaccharides.

Nonetheless, the amount of polysaccharide that was potentially removed from white potato peels was significantly lower than the amount of biomass loss. This suggests that the losses are not only due to enzymatic degradation, and are possibly associated with the washing process or the scraping of the filters after the digestion. HPLC results present a deviation of about 10% from bibliographic findings for polysaccharide content of tomato skin and white potato skin (≈19% in HPLC contrary to 30% described in the literature) which could be explained by the variance of polysaccharide content between potato strains. The phenol-sulfuric colorimetric method was proven to be unreliable because it resulted in unreproducible polysaccharide content, which might be due to other carbohydrates being quantified (Nielsen, Science, & Series, 2010). Also, more monosaccharide standards could have been used in HPLC analysis, such as mannose, arabinose and xylose, to assure a more complete quantification of all polysaccharides in the peels, given only glucose, fructose, ribitol and sorbitol standards were used for quantification.

ATR FT-IR analysis suggests a diminished contribution of polysaccharides (peaks between 1207 and 865 cm^{-1}) in both tomato and white potato skin. Although we should keep in mind that the ATR FT-IR analysis is merely qualitative and related to the surface of the sample, the fact that several readings of the enzymatically digested biomass reveal smaller polysaccharide related peaks is highly suggestive of their reduced concentration.

Concerning the amount of suberin that was possible to recover through cholinium hexanoate-mediated extraction, yields were lower than with previous findings (Ferreira et al., 2014) – less than 50% of suberin was obtained from cork with the extraction lasting 2 hours, and suberin extraction from white potato skin yielded around 3.5%. However, previous experiments leading to much higher suberin yields carried out extractions with agitation and for longer periods, which might justify these low yields. Cutin yield from tomato skins were around 5.6% for untreated biomass and only around 0.6% for enzymatically hydrolyzed source. This suggests that either the enzymatic hydrolysis and/or the Soxhlet extraction are not adequate for the tomato skin. However, some factors must be considered regarding the usefulness of the treatments. For one, cutin is present in a more superficial layer of the fruits, meaning successive

Soxhlet extractions could eventually drag polymer along. Another consideration is that cutin is usually white, and the one obtained from untreated tomato peels turned out yellow, possibly indicating either the oxidation of the compounds or the presence of polysaccharides.

In addition to lower yields, mass balances resulting from summing the insoluble residue mass with that of the biopolymer revealed significant losses of mass that are not entirely understood. These losses were of 15% and 25% for the extraction of suberin from cork for 1 hour and 2 hours respectively, 41.5% for white potato skin, 25.6% for cutin extraction from untreated mass and 12.6% for hydrolyzed mass. This biomass may have been lost during the successive washes and centrifugations that follow the extraction step.

Also, biopolymer and insoluble residue ATR FT-IR characterization reveals that some biopolyester is still present in the insoluble residue. This supports the fact that the extraction, although effective, was not complete, likely because of insufficient mixing of ionic liquid and biomass, leading to poor homogeneity: extractions were done without any agitation mechanism and some of the biomass might have not been adequately dissolved in the ionic liquid catalyst.

In relation to the GC-MS analysis, suberin extracted from 2 different sources expectedly revealed an overall similar composition, with the two most abundant family of compounds being hydroxy fatty acids (mono) and alkanedioic acids, although in different percentages (Table 1.9 in Appendix). However, calculated percentage of fatty acids and fatty alchools in suberin from potato skin are very similar to that of cutin from tomato skin (3.40% and 3.50%, and 0.64% and 0.31% respectively). Literature review on content of monomers in suberin from *Quercus suber* (Ferreira et al., 2014) reveals some differences in the percentage of monomers, yet revealing similar results in the most abundant monomers, namely 22-hydroxydocosanoic acid and 9,10-dihydroxyoctadecanedioic acid. On the other hand, published work on GC-MS of potato periderms corroborate 18-hydroxyoctadec-9-enoic acid as one of the major monomeric components in suberin (Graça & Pereira, 2000). Low contents of glycerol in all samples of the ionic liquid recovered suberin have been previously described (Ferreira et al., 2014). This is due to an extensive cleavage of the acylglycerol bonds of the biopolyester during the extraction with cholinium hexanoate. Glycerol is normally the most abundant monomer in suberin (Helena Pereira, 2015), yet the cholinium hexanoate extraction causes the release of glycerol molecules that are soluble in water, hence lost during the precipitation of the biopolyester in water.

As for cutin, GC-MS experiments from previous publications reveal 10,16-dihydroxyhexadecanoic acid as the main monomeric unit in this biopolyester (Cifarelli, Cigognini,

Bolzoni, & Montanari, 2014) whereas hexadecanoic acid was the major contributor in the analysis performed in this work.

Overall, the differences in monomeric compositions of the biopolyesters extracted are likely due to the extraction method applied, given most literature relied on alkaline hydrolysis to obtain the monomers.

CHAPTER 2 | Hybrid Materials in Gas Sensing

2.1 Introduction

In this second chapter, the application of suberin and cutin in the production of hybrid materials will be presented and discussed. At Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa (FCT-NOVA), the Biomolecular Engineering Group has developed an E-Nose with the purpose of detecting and discriminating volatile organic compounds (VOCs). Although still under improvement, the device can detect optical changes in the LC and convert it to an electrical signal response through time whenever a film is exposed to VOC molecules.

So far, the most promising films tested consist in LC-IL droplets embed in a polymeric matrix, resulting in a stable hydrogel (Hussain et al., 2017). These droplets resulted from the cooperative self-assembly of 5CB with the ionic liquid 1-butyl-3-methylimidazolium dicyanamide [BMIM][DCA] in the presence of water, causing the LC to organize in a radial form, and the matrix resulted from the dissolution of gelatin in [BMIM][DCA]. The sensing mechanism of the resulting hybrid material relied on the ability of the LC to rotate the plane of polarized light and its reversible disruption when a certain VOC is present. This property of the liquid crystal is what makes it a great sensing material (A. Hussain et al., 2009).

While the main idea is that the polymer's only role is to stabilize the LC-IL droplets, preventing it from dispersing in the matrix, this thesis's proposition is to test hybrid materials in the form of films without ionic liquid and evaluate its stimuli-responsive potential to VOCs.

2.1 Materials and Methods

Biopolymers

The biopolymers used to produce the sensing films were the ones obtained through the extraction method described in Chapter 1. They are suberin from 30-minute, 1-hour and 2-hour extraction from refined cork, suberin from 2-hour extraction of untreated white potato skin and cutin from 2-hour extraction of untreated tomato skin. Gelatin from bovine skin, 1-docosanol and 16-hydroxyhecadecanoic acid used for control tests were bought from Sigma-Aldrich.

Other chemicals

The liquid crystal 4-cyano-4pentylbiphenyl (5CB) was bought from TCI Europe, n-Hexane (95%) was bought from ThermoFisher (VWR), Toluene (pure), Dichloromethane (>99%) and Ethanol (absolute) were bought from PanReac AppliCHem and Acetone (100%) was bought from LabChem.

E-Nose

The version of the E-Nose used (Fig. 2.1) comprises a closed chamber with a platform consisting of four light dependent resistors (LDR), a LED-light source for each one and, in the middle, four slots where the films are inserted. Between the LDR and the slots, and the LED sources and the slots, there are two polarizers, perpendicular to each other. The exposition and recovery pumps had a constant flow of 4.37L/min and 3.0L/min, respectively.

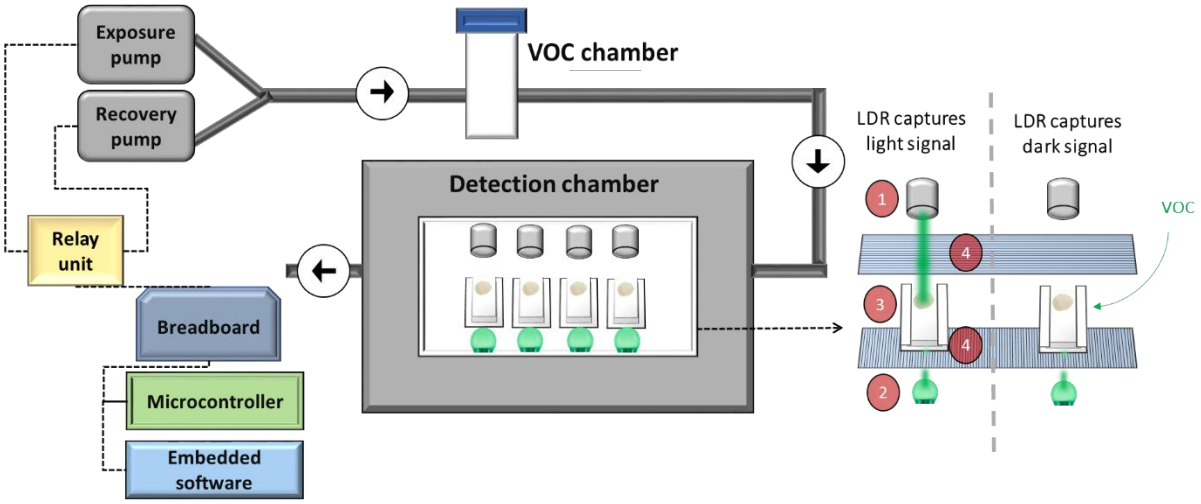

Figure 2.1 - Schematic representation of the E-Nose used in this work; (1) LDR; (2) LED-light; (3) Sample slot; (4) Polarizers.

Film production

The production of the first films followed a protocol already established for the formulation of gas-sensitive films based on gelatin from bovine skin (Appendix Figure 2.44). In this case, gelatin was replaced by suberin from 30-minute extraction of cork. The second type of films produced had the following general formulation: **Biopolymer + H2O + 5CB**, where the ionic liquid was removed.

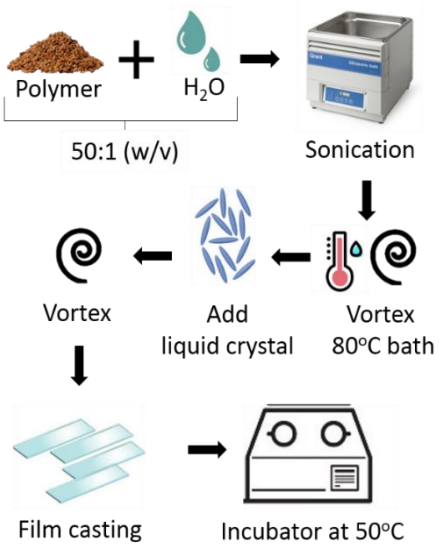

Figure 2.2 – Film production process.

The method for film production needs to be adapted for each biopolymer under study. Before starting the production of the films, glass blades had to be cut to 40x15mm dimensions to fit the E-Nose sensor chamber. All glass blades were previously marked so that every film was cast in the same place. Firstly, 1.3mg of biopolymer were weighted and mixed with 65µL of distilled water in a 0.5mL eppendorf (2% suberin w/v). This mixture was sonicated for 2h with cycles of 30 minutes (80W; 220-240V) then further mixed in a vortex for 5 minutes and put in an 80°C bath while under 700 rpm

agitation for 45 minutes with a magnetic stirrer. Then, 3.0µL of 5CB were added to the hot mixture, which then went to vortex for another 10 minutes. Lastly, 5.0µL of the final mixture were pipetted to the glass blades without spreading, and these were kept for 15 minutes in an incubator previously heated to 50°C. The various formulations produced and tested are listed in Table 2.1.

Table 2.1 – List of all film formulations used for E-Nose tests on VOC exposure.

Biopolymer	Code	Alterations to general protocol	# Replicates	Replicates tested
Suberin from 30-min extraction of cork	29A	+15min 80°C bath	6	29A1 29A5
Suberin from 1-hour extraction of cork	29B	+15min 80°C bath	4	29B2 29B3
Suberin from 2-hour extraction of cork	13#	+30 min sonication	6	13B 13D
Suberin from white potato skin	20P	-	8	20P3 20P4
Cutin from tomato skin	TC	+ 4 hours sonication; 80°C bath and 700rpm vortex used alternately for 30min	8	TC2 TC4
Controls	**Code**	**Alterations to the general protocol**	**# Replicates**	**Replicates tested**
1-Docosanol	D22	Replacement of test-biopolymer for suberin monomer 1-docosanol	3	D22B D22C
Gelatin from bovine skin	GH	Replacement of test-biopolymer for gelatin	4	GH2 GH4
Liquid crystal	LC	No film; only 1 drop of LC	2	LC1 LC2
No Liquid crystal	SubH	Only suberin from 2-hour extraction of cork and water	6	SubH6

The controls were selected based on the following rationale: 1-Docosanol is a long-chain monomer present in suberin (C_{22}) from the family of Alkanoic acids; 16-hydrohexadecanoic acid is a small-chain monomer from the family of hydroxylated fatty acids (but no films were possible to produce with the protocol implemented); Gelatin from bovine skin is the standard polymer used to produce hybrid films for the E-Nose developed at FCT-NOVA; Liquid crystal droplets acted as positive controls due to their role as optical probes in hybrid materials. Hence, by comparison with the other films, it is possible to extrapolate the contribution of the liquid crystal alone for the overall optical response of the hybrid material. Finally, the control without liquid crystal is used as negative control because it allows the evaluation of the response of suberin alone when exposed to VOCs.

Morphological characterization

The morphological characterization was made recurring to Polarized Optical Microscopy (POM), Scanning Electron Microscopy (SEM) and AFM (Atomic Force Microscopy). POM images were taken throughout time to evaluate the films' stability to storage, prior and after each exposure to the volatiles, with a Zeiss Axio Observer.Z1 polarized optical microscope, coupled with a Axiocam 503 color camera. Using the ZEN 2.3 processing software, this microscope allowed photographing regions of the films to make a visual analysis of the homogeneity of the material, and later compare their morphological resistance to the different volatiles. All the POM photos presented here have a 50x magnification, resulting from the ocular amplification (10x) multiplied by the objective amplification (5x), and all samples were analyzed under crossed polarizers.

AFM images were obtained at CENIMAT (FCT-NOVA) on an Asylum Research MFP-3D Standalone System, equipped with Olympus AC160TS (f0=300 kHz; k= 26 N/m) tip, and image processing was made using Gwyddion – SPM data analysis software. The purpose of this analysis was to scan for micro or nanostructures that the biopolymer could developed when in contact with liquid crystal. For this, a film containing suberin from the 2-hour extraction from cork (13# formulation) and a sample of that same suberin but without 5CB (SubH formulation) were analyzed.

SEM images were obtained at CENIMAT (FCT-NOVA) on a Zeiss Auriga CrossBeam Workstation equipped with a FIB column. Along with AFM analysis, the objective was characterizing the morphology of the surface of films with different polymers. The samples analyzed were TC, GH, D22, 13#, SubH and 20P formulations. Before entering the column, samples had to be dried and coated with 20µm of AuPd (40% and 60% respectively) for better conductivity and placed on a carbon-aluminum support.

Exposure of hybrid materials to VOCs

The exposure of the hybrid materials/films produced to VOCs was performed on an E-Nose developed by the Biomolecular Engineering group. For the VOC sensing tests, the following compounds were tested, in the order they are listed:

- N-Hexane (95%) – Vapour pressure: 248.8mmHg (37^{o}C)
- Toluene (pure) – Vapour pressure: 51.1mmHg (37^{o}C)
- Dichloromethane (pure) – Vapour pressure: 696.9mmHg (37^{o}C)
- Ethanol (absolute) – Vapour pressure: 115.7mmHg (37^{o}C)
- Acetone (>95%) – Vapour pressure: 377.7mmHg (37^{o}C)
- Water (plain) – Vapour pressure: 47.1 mmHg (37^{o}C)

The tests in the electronic nose were performed under constant conditions for each formulation and VOC. Two films of each formulation were exposed twice to 5 different VOCs in increasing order of polarity – Hexane, Toluene, Dichloromethane – and the final two were Ethanol and Acetone whose order got switched because, despite the higher polarity of Ethanol, Acetone is suspected to damage the films, while Ethanol is usually harmless. The conditions of each test were as follows:

- Volume in flask: 5mL
- Time of exposition: 60 seconds
- Time of recovery: 100 seconds
- Sampling frequency: 40hz
- Duration of sampling: 30 minutes
- Temperature of the room: 22ºC
- Temperature of the sample: 37ºC
- Atmospheric pressure: 760 mmHg
- Humidity: 30%
- Exposure flow: 4.37L/min
- Recovery flow: 3.0L/min

The data obtained was analyzed by Principal Component Analysis (PCA) using the Orange Canvas software considering the following features: relative amplitude of the signal (a) x and y coordinates of the maximum signal (x and y); maximum/minimum of the 1st derivative of the signal (d) and the time it takes the film to respond to the VOC (t) (Figure 2.3). Besides PCA, the average amplitudes of the signals produced by sample films were also discussed, given they make it easier to access the intensity of the response. For control tests, only the average amplitude was considered.

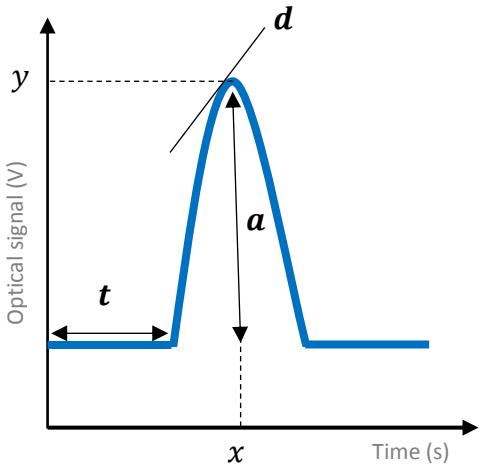

Figure 2.3 – Representation of the features used for PCA analysis of E-Nose responses after VOC exposures.

Live VOC exposure – Polarized optical microscope VOC chamber

Live VOC exposures were filmed with the same POM microscope using a glass chamber designed and built at FCT to observe what happened to the film in the precise moment the VOC interacted with the matrix. Only one film from the 20P formulation was observed.

For these tests, 5mL of each volatile were heated in a glass vial, sealed with a rubber lid, up to 37ºC for 10 minutes. After that, a 10mL syringe coupled with a needle was used to perforate the lid and 10mL of volatilized liquid were collected. The syringe was sealed and transported to the room were the VOC chamber was installed, and the volatile was pumped into

the chamber where the sample was trapped. For acetone and ethanol, an air pump was not required to remove the VOC from the chamber after exposure given these liquids are not highly dangerous to human health when volatilized. For dichloromethane, hexane and toluene, an air pump was necessary because the chamber is not perfectly sealed and these volatiles were easily perceived in the ambient air after pumped inside the chamber. In addition, the pumping of the volatiles was not ordered in the same way as the E-Nose tests. A schematic representation of the VOC chamber is present below (Figure 2.4)

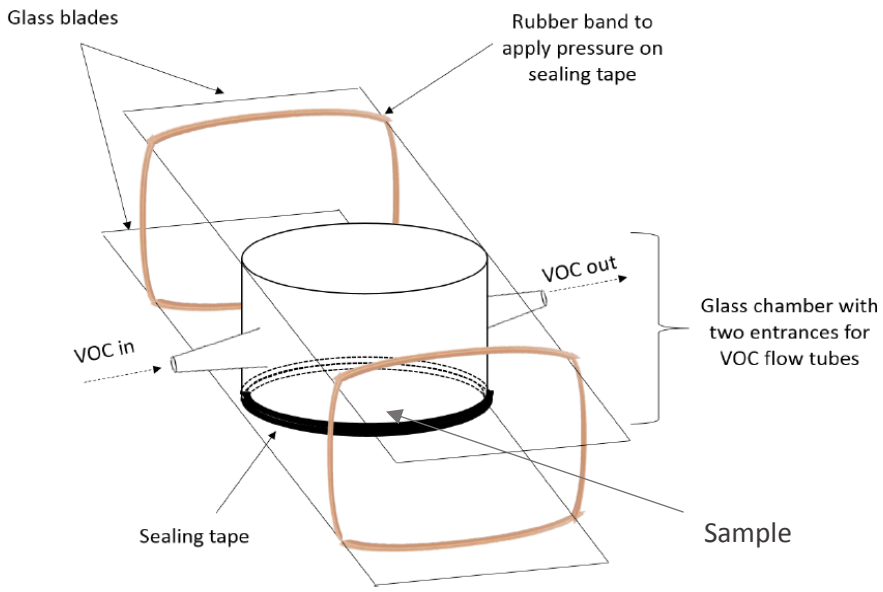

Figure 2.4 – Schematic figure of the live-VOC testing chamber.

2.2 Results

Film production

The first films produced following the protocol already established for the production of hybrid gels (Appendix Figure 2.44) with replacement of the gelatin from bovine skin with suberin from 30-minute extraction of cork resulted in an insolubilized suberin matrix with liquid crystal dispersed amid (Figure 2.5B). This highly contrasted with the standard films using gelatin, which resulted in gel-like materials with micelles trapped in a stable solubilized gelatin matrix (Figure 2.5A).

After several attempts to produce micellar structures, which included dissolving suberin in cholinium hexanoate and dispersing the film through hand-roll method (Figure 2.5C) and spin coating method (Figure 2.5D), sonicating suberin in ionic liquid prior to LC addition, neither experiments resulted in stable films (Appendix Figures 2.19-2.20) and the best solution ended up being the discarding of the ionic liquid and production of hybrid material following the general formulation presented previously.

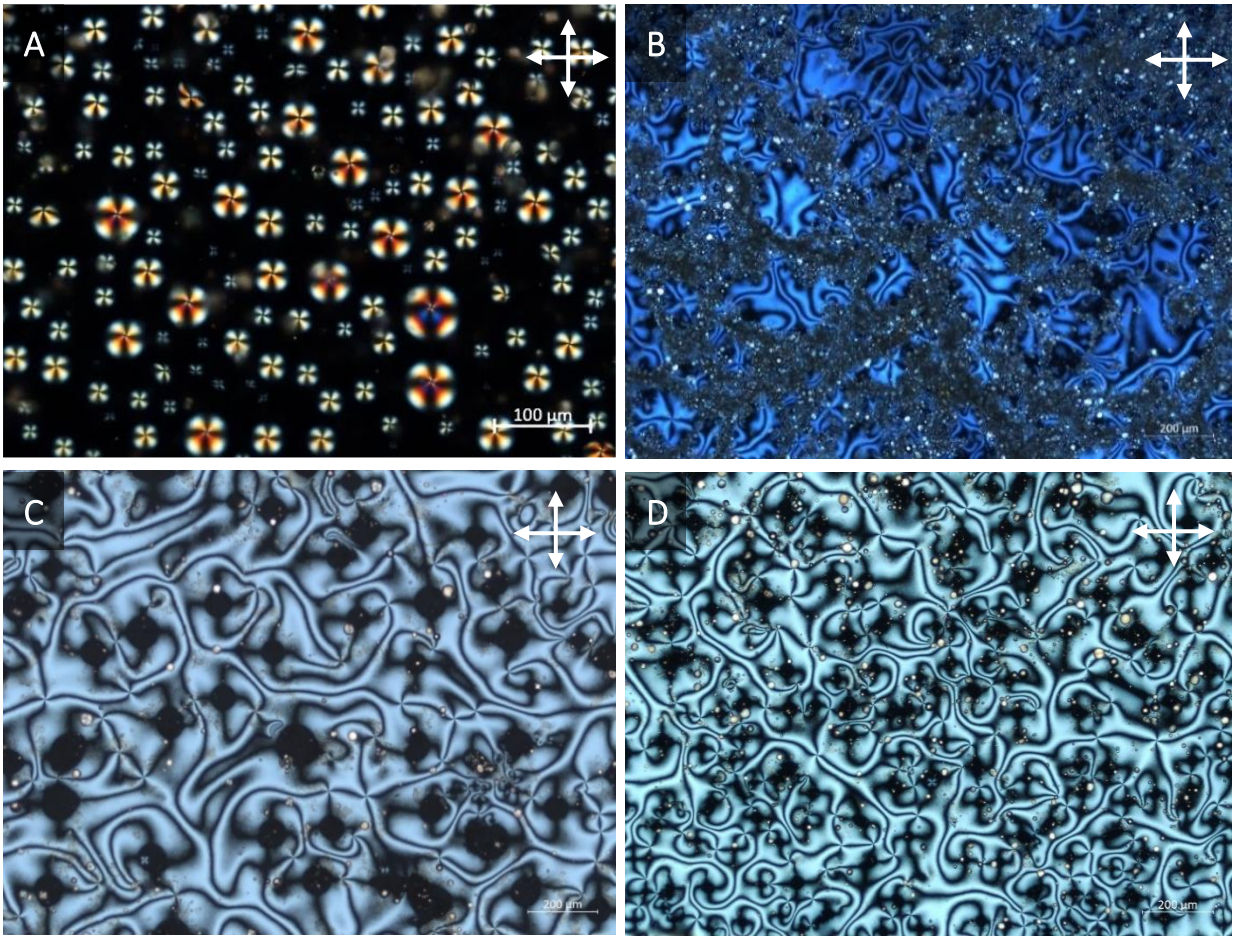

Figure 2.5 - POM images of A: film with gelatin + [BMIM][DCA] + 5CB; B: film with suberin from 30-minute extraction of cork + [BMIM][DCA] + 5CB; C: film with suberin + CH + 5CB casted with hand-roll method; D: film with suberin + CH + 5CB casted with spin coating method. 50x magnification.

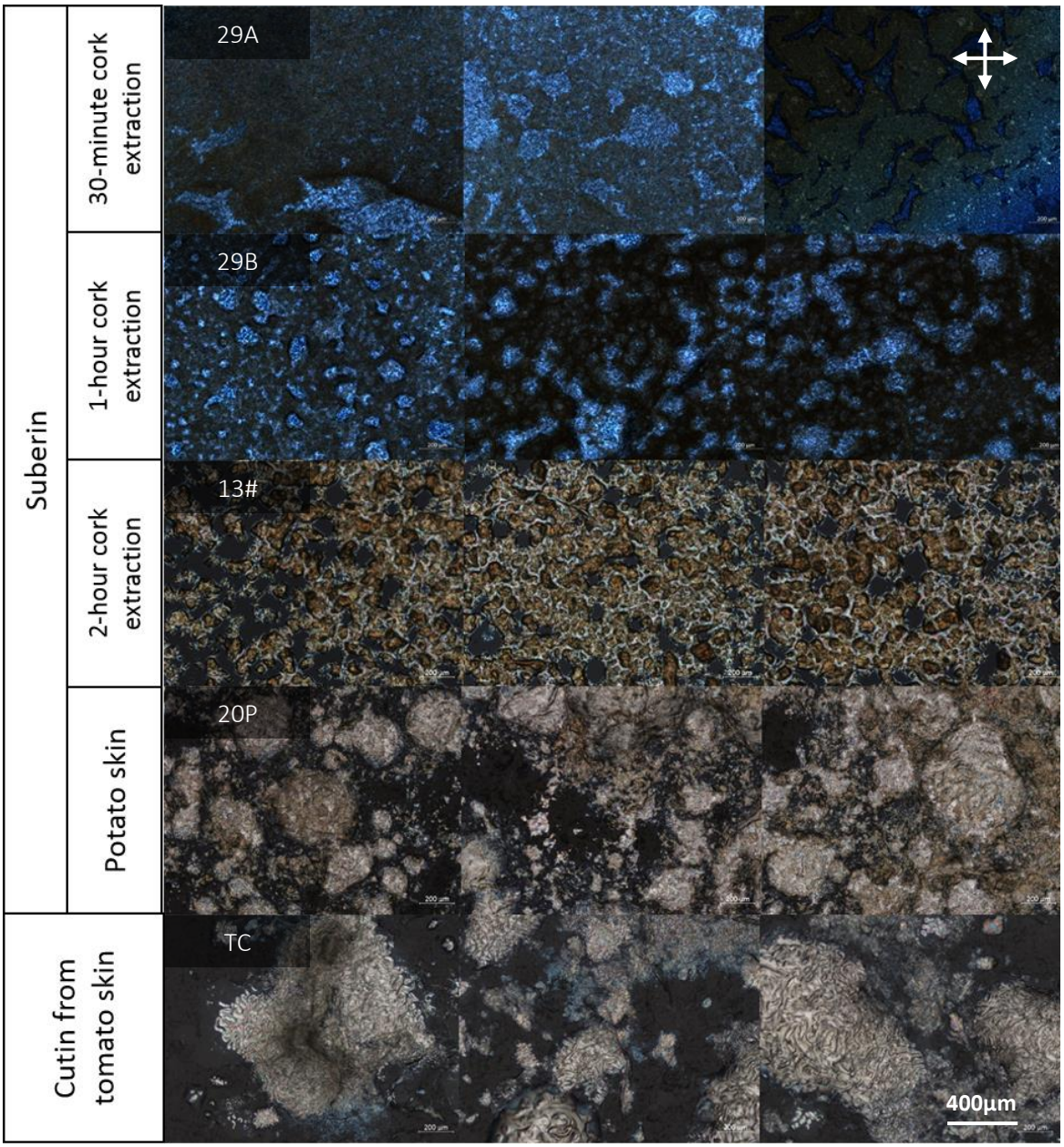

Figure 2.6 – POM images of biopolyester based-films produced for E-Nose tests (3 examples each). General formulation used: Biopolymer + H2O + 5CB.

The films depicted are not the totality of films produced. For that information, consult the appendix Figures 2.21 to 2.34.

The films produced with suberin extracted from cork for 1 and 2 hours show homogenous and reproducible morphologies, although both formulations appear very different from each other. Overall, the films produced with suberin from 1-hour extraction of cork (29B) reveal a more dispersed suberin with LC filling in the empty spaces, while films produced with suberin from 2-hour extraction from cork (13#) reveals a more fragmented suberin with LC preferentially around suberin structures and more empty spaces within the film. Films with suberin from 30-minute extraction of cork resulted in highly heterogenous morphologies and, overall, there

seems to be less empty spaces within the film, with LC preferentially deposited around the film. The production of films incorporating suberin from white potato skin reveal a significantly different morphology in comparison to cork suberin-based films. Nevertheless, the formulation is reproducible and suberin appears to cover the LC.

Tomato cutin-based films did not reveal neither homogenous morphology nor reproducibility. The LC appears highly intertwined with the biopolymer matrix, but a lot of the film has no LC. Also, of all films produced, the TC formulation revealed the weakest consistency, while other formulations were stable and dry.

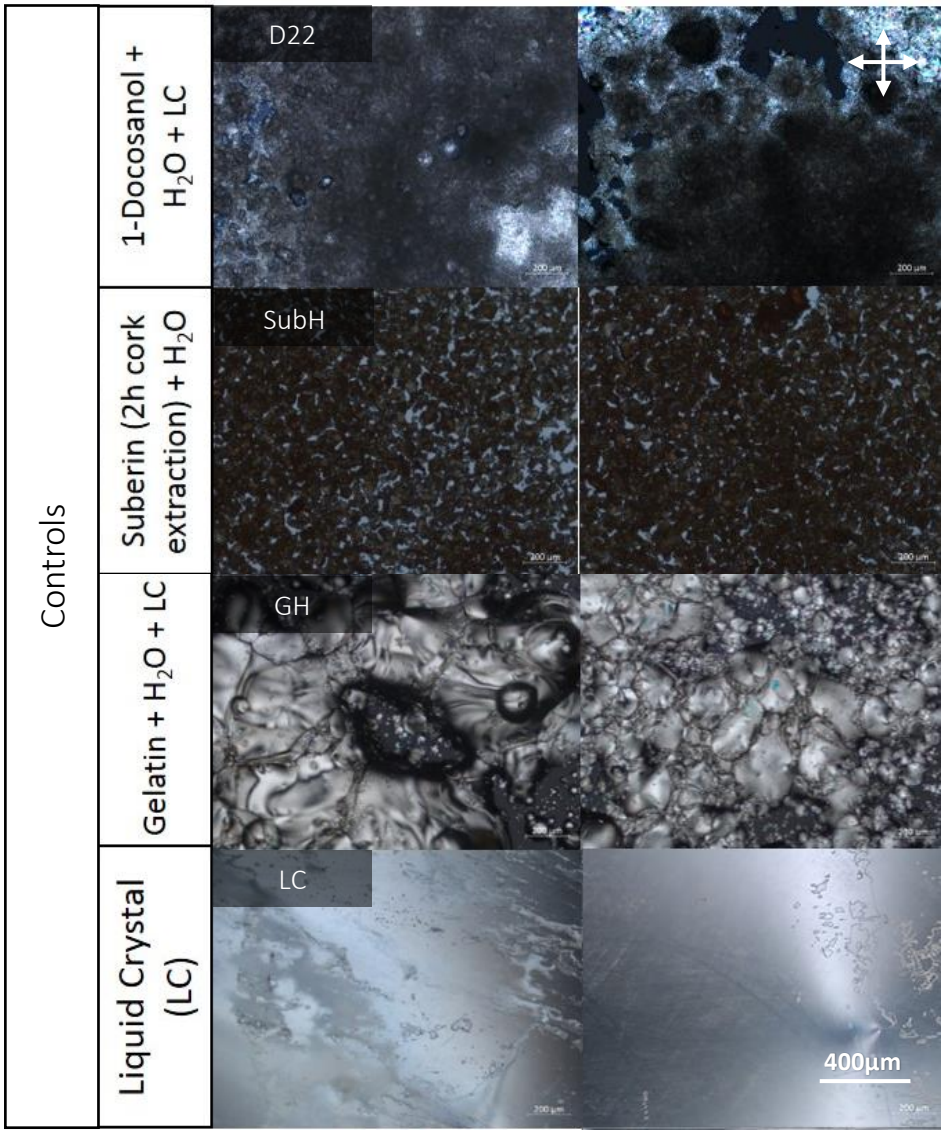

Figure 2.7 – POM images of the control films produced for E-Nose tests (2 replicates each).

As for the control formulations, D22 shows a highly heterogenous morphology and reproducibility was inexistent, given 2 of 3 films remained with a paste-like texture and high instability. The suberin and water control (SubH) led to highly homogenous and reproducible films. The pattern of suberin organization is similar to that of 13# formulation but with less empty space between suberin structures. The gelatin-based control lead to the formation of micellar-like structures incorporating the LC inside. All replicates were moderately homogenous and reproducible. Finally, the LC control revealed the drops of liquid crystal organize similarly when isolated from other components.

Morphological characterization

- **Storage stability**

Stability to storage was only tested for 29A and 29B films.

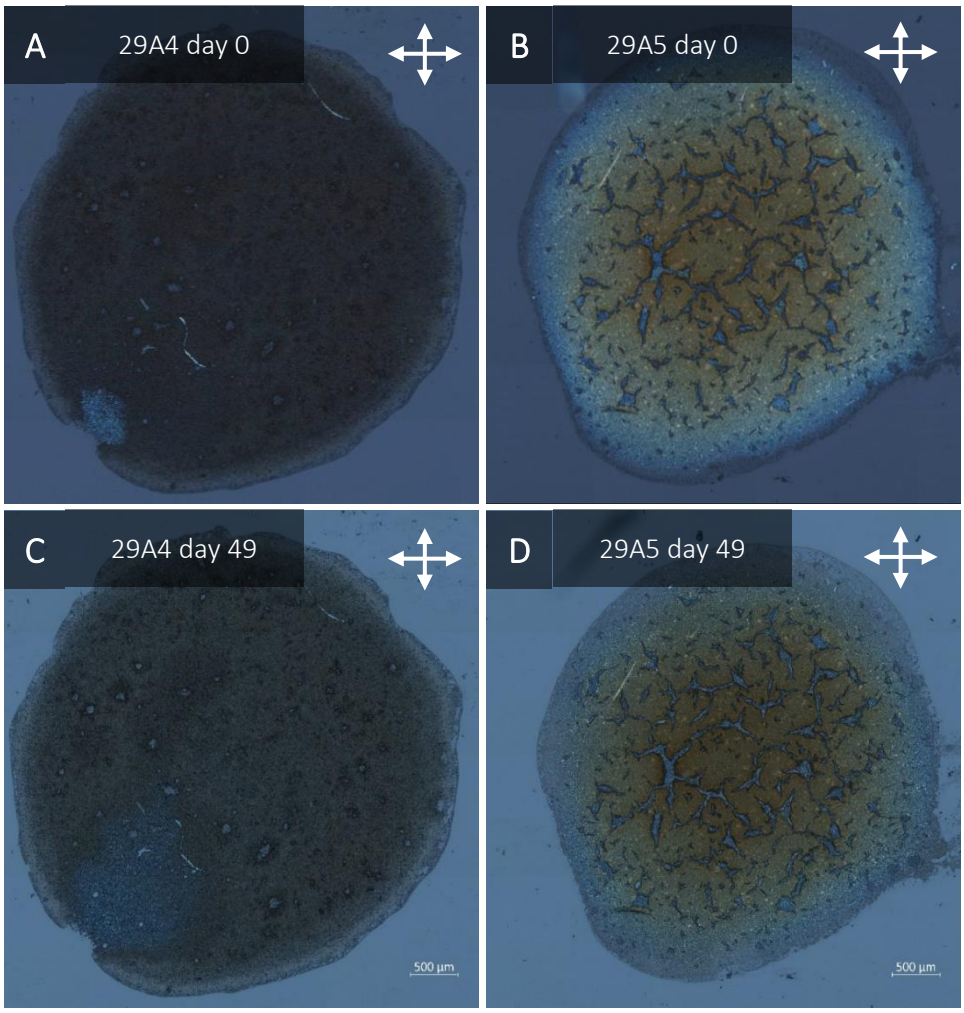

Figure 2.8 - POM images of two films produced with suberin from 30-minute extraction of cork + H2O + 5CB after production, 29A4 and 29A5, (A and B, respectively) and 49 days later, before exposure to VOCs in the E-Nose (C and D respectively).

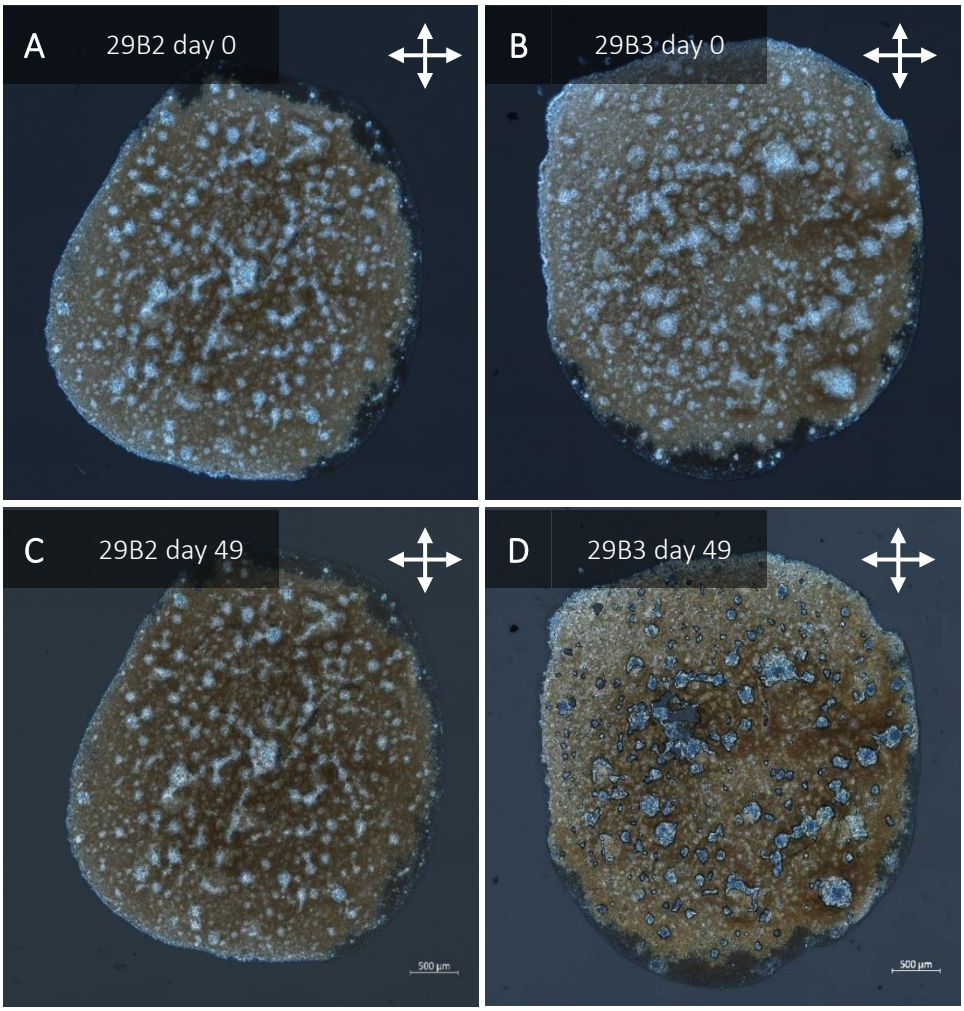

Figure 2.9 - POM images of two films produced with suberin from 1-hour extraction of cork + H2O + 5CB after production, 29B2 and 29B3, (A and B, respectively) and 49 days later, before exposure to VOCs in the E-Nose (C and D respectively).

The replicates for both formulations (29A and 29B) did not show significant changes in their morphology after 49 days stored in a sealed petri dish. In the 29B3 film, 49 days after production, the LC visible under POM is less in amount, but it should be held in consideration that the film was exposed to E-Nose tests, although with irregular and/or insufficient concentration of volatiles in the E-Nose chamber due to homogeneity problems associated with the device.

- **Resistance to VOC exposure**

All films exposed to VOC testing except those based on cutin (TC formulation) and isolated liquid crystal drops (LC formulation) showed no significant changes in their morphology by the end of each test. In general, the unchanged films only presented a slight modification in the texture of the liquid crystal after exposure, but this was regarded as insignificant due to unaltered optical responses following these observations. POM images of the films that underwent VOC exposure are present in the Figure 2.10.

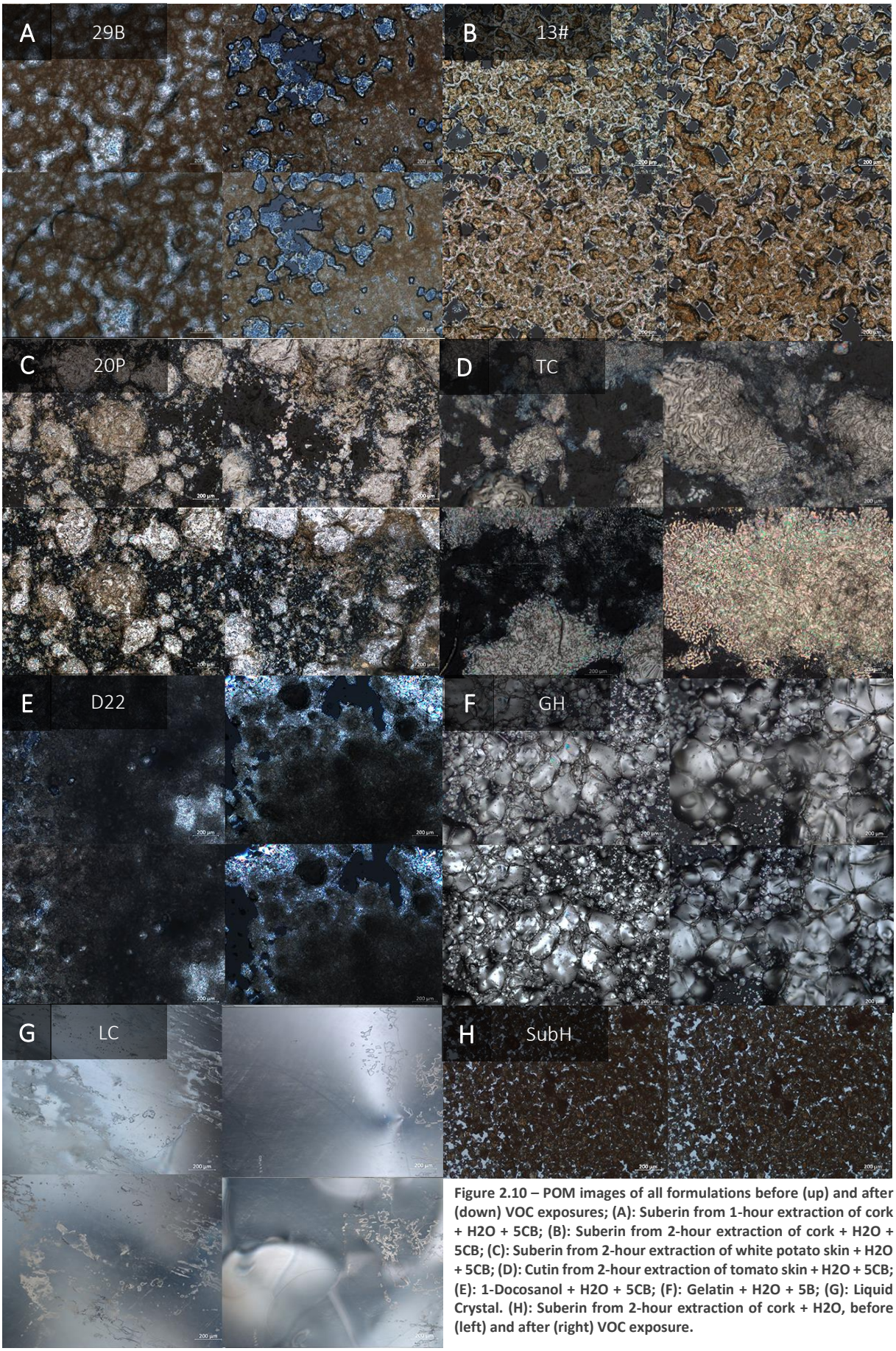

Figure 2.10 – POM images of all formulations before (up) and after (down) VOC exposures; (A): Suberin from 1-hour extraction of cork + H2O + 5CB; (B): Suberin from 2-hour extraction of cork + H2O + 5CB; (C): Suberin from 2-hour extraction of white potato skin + H2O + 5CB; (D): Cutin from 2-hour extraction of tomato skin + H2O + 5CB; (E): 1-Docosanol + H2O + 5CB; (F): Gelatin + H2O + 5B; (G): Liquid Crystal. (H): Suberin from 2-hour extraction of cork + H2O, before (left) and after (right) VOC exposure.

SEM analysis

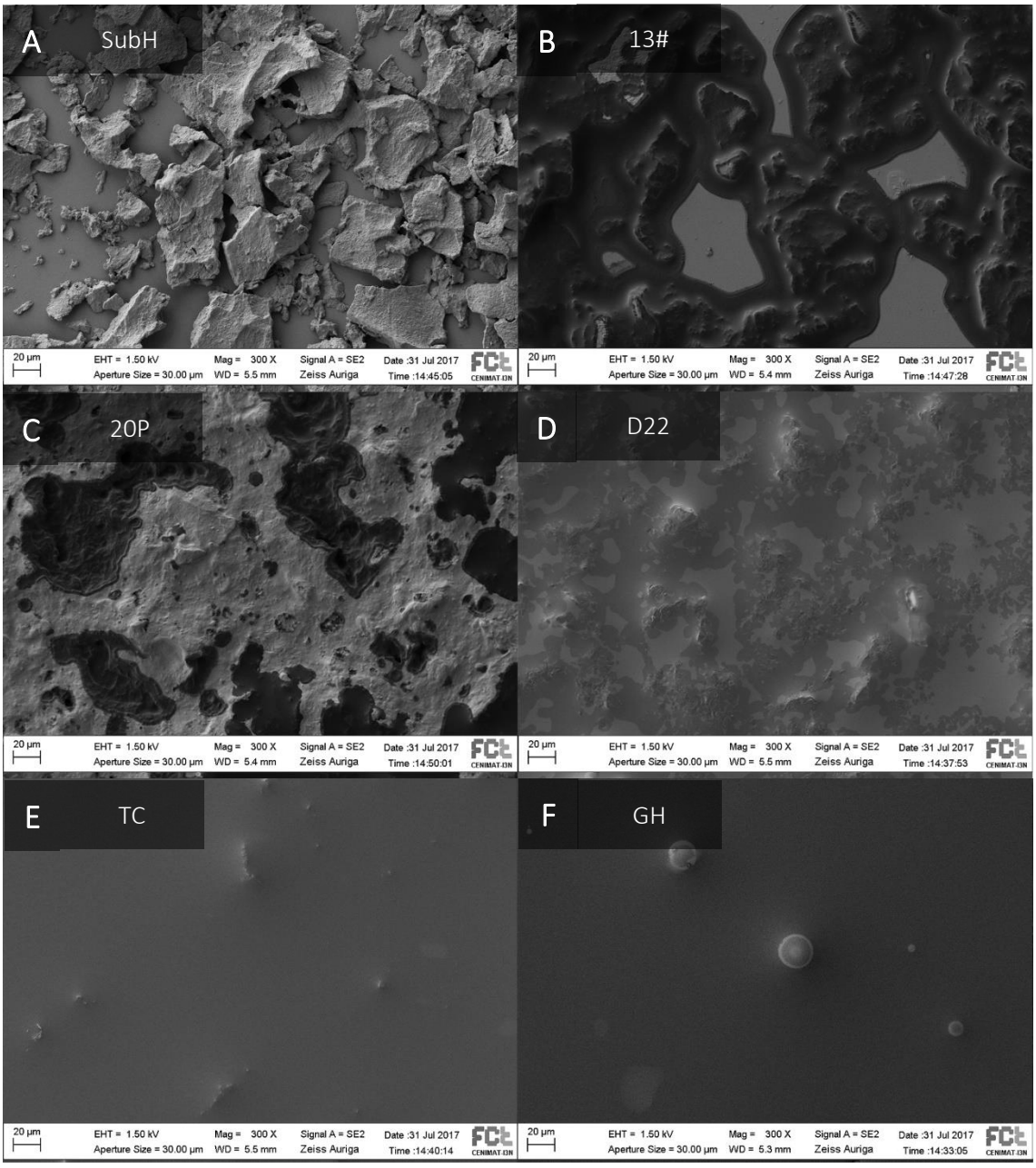

Figure 2.11 - SEM images from formulations (A) Suberin from 2-hour extraction of cork + H2O; (B) Suberin from 2-hour extraction of cork + H2O + 5CB; (C) Suberin extracted from white potato skin + H2O + 5CB; (D) 1-Docosanol + H2O + 5CB; (E) Cutin from tomato skin + H2O + 5CB and (F) Gelatin + H2O + 5CB.

SEM images reveal substantial differences between all formulations. Surprisingly, suberin-based films do not look alike, and the ones containing 5CB appear to have what looks like a coating above suberin molecules, making the marked edges and light geometry of suberin look soften in the case of 13# formulation. In the 20P sample, 5CB and suberin appear almost indiscernible in terms of texture, with the only differences being the color and surface protrusion levels.

As for the GH formulation, even though it did not contain [BMIM][DCA], SEM revealed circular structures, suggesting a high structural difference from suberin-based films. The TC film also provided completely different SEM images when comparing to suberin. Its surface appears much more homogenous and the polymer structures are almost undetectable, whereas in 20P and 13# formulations some rough texturization is visible.

AFM analysis

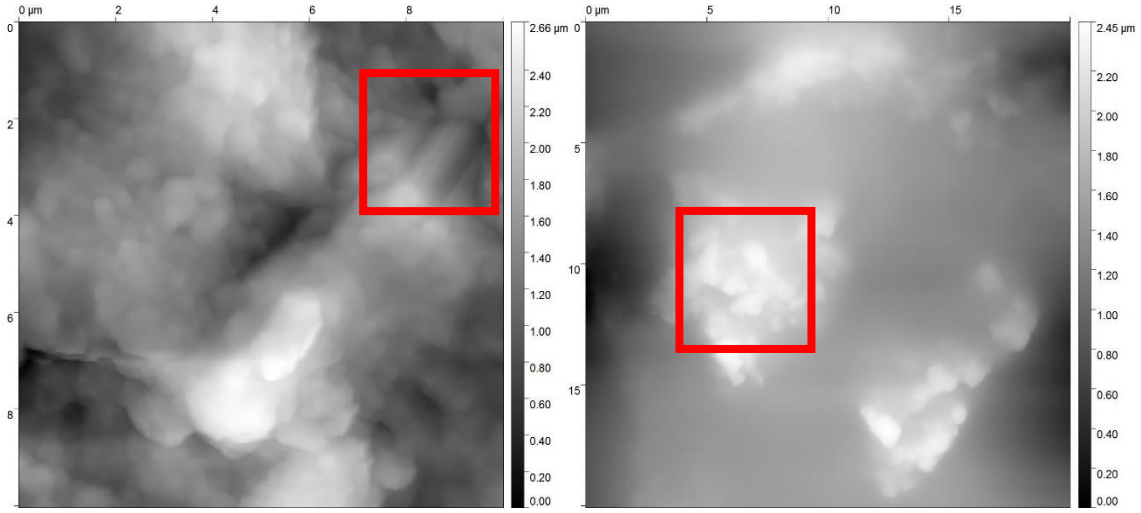

Figure 2.12 - AFM images from formulations (A) Suberin from 2-hour extraction from cork + H2O (SubH) and (B) Suberin from 2-hour extraction from cork + H2O + 5CB (13#.)

AFM images contain less detail, but these results seem to corroborate SEM analysis regarding the coating effect the 5CB seems to cause. Some orientation or geometry of suberin molecules can be perceived, especially in the areas outlined in red. It also appears that suberin does not lose much of its configuration when in presence of 5CB, which might indicate that, molecularly, they do not interact significantly.

Exposure of hybrid materials to VOCs

- **Control tests**

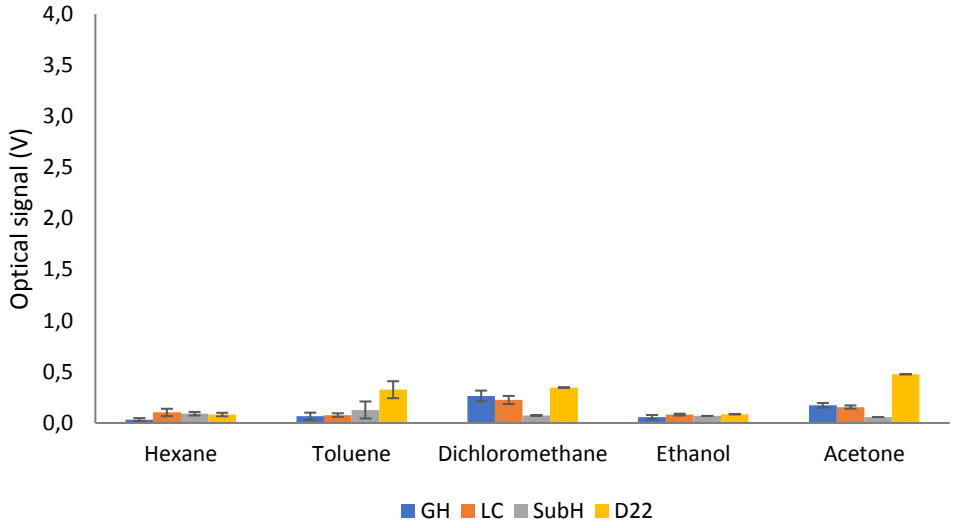

Figure 2.13 – Average amplitude ranges of all control formulations to all the VOCs tested.

Control tests reveal that, overall, neither film respond well to the volatiles used. The D22 films respond slightly better to acetone, dichloromethane and acetone, while showing near-baseline responses to hexane and toluene like the other three control formulations. Regarding reproducibility of optical responses, appendix Figures 2.40 to 2.43 suggest high reproducibility in all four formulations, with a slight deviation from baseline occurring during GH4's exposure to dichloromethane, probably due to punctual malfunction of the E-Nose.

- **Sample tests**

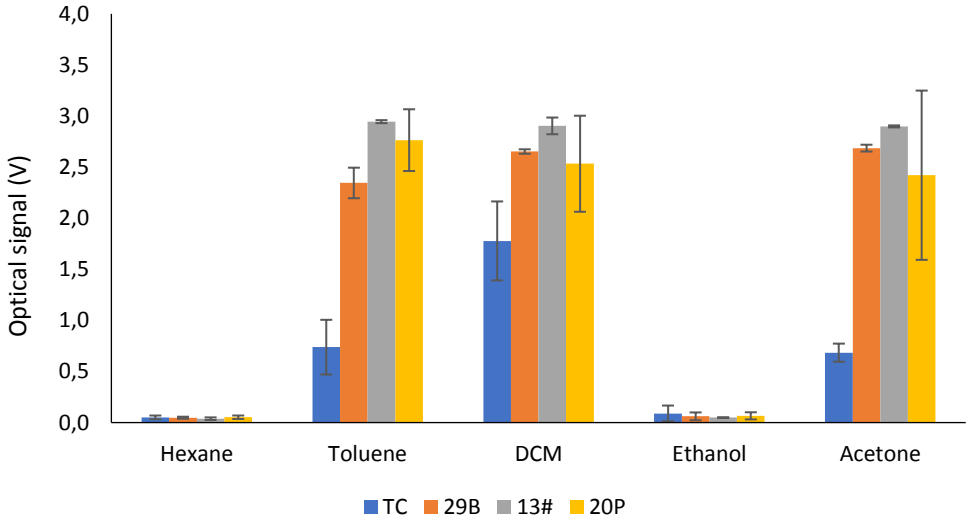

Figure 2.14 - Average amplitude ranges of all sample formulations to all the VOCs tested.

As for sample formulations, data relative to the average amplitude range of the electrically converted optical signal reveals that suberin-based films (29B, 13# and 20P) have the tendency to higher optical responses to toluene, dichloromethane and acetone, while responding poorly to hexane and ethanol. Of all suberin-based films, those containing suberin from white potato skin (20P) show a much higher standard deviation and those containing suberin from 2-hour extraction from cork (13#) show the most reproducible responses. Cutin-based films (TC) produce an overall much lower optical signals and high standard deviations, while responding similarly to suberin-based films to hexane and ethanol.

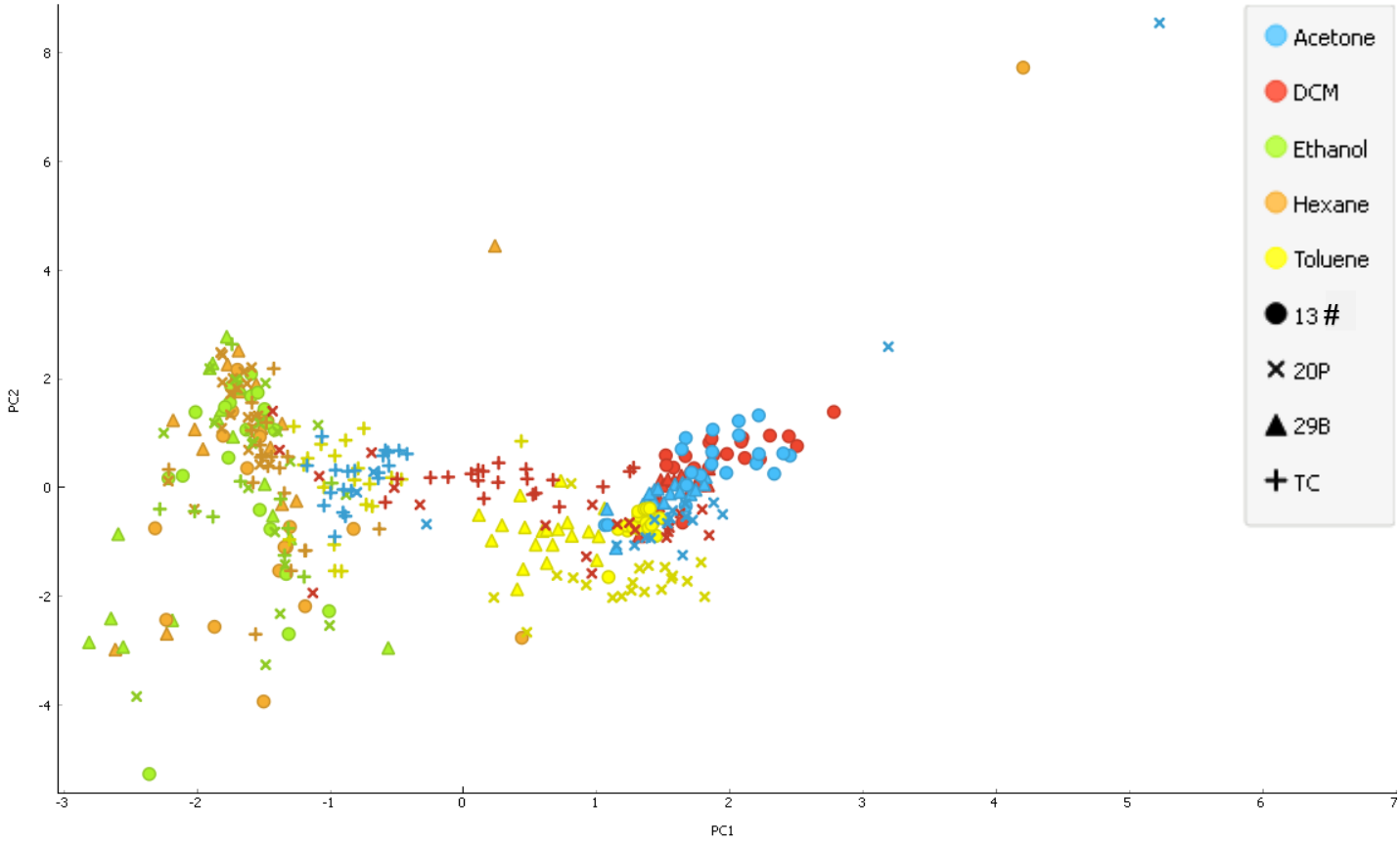

Figure 2.15 – PCA analysis done on data from VOC exposures of films containing suberin from 1-hour extraction of refined cork (29B), suberin from 2-hour extraction of refined cork (13#), suberin from white potato skin (20P) and cutin from tomato skin (TC) in the E-Nose.

PCA analysis shows that there are two main clusters – to the left responses to ethanol and hexane; to the right responses to acetone, dichloromethane and toluene. Data relative to responses from suberin-based films (13#,20P and 28B) reveal their low capacity to discriminate between acetone, dichloromethane and toluene, but a high capacity to discriminate between those and ethanol/hexane. Nonetheless, there are some differences among suberin-based films. 29B formulation seems respond differently to toluene than the other formulations, while still showing the same tendency to not discriminate between ethanol and hexane.

The results from the 29A formulation are present in the appendix (Figure 2.35 in Appendix) and only serve as an example of an unsuccessful film production, given it resulted in extreme heterogeneity that severely compromised the comparison with the other formulations.

Cutin-based films show no particular affinity to neither of the tested volatiles, represented by the fact that they do not preferentially gather in any of the clusters.

Regarding reproducibility of the optical response, results present in the appendix (Figures 2.36 to 2.39) show that the 29B, 13# and TC formulations are reproducible within technical and biological replicates, while 29A and 20P are only reproducible within technical replicates. The significant decrease of the optical signal closer to the end of the tests is very likely related to the complete evaporation of the volatile in question.

Water exposure tests

Water exposure tests are crucial to avoid incorrect optical readings, because films should be unsensitive to water, given the humidity present in most samples to be tested in the future carry some humidity (e.g. foods, human breath). The tests were performed on the previously exposed films from 13#, D22, GH and LC formulations and amplitude ranges reveal that none of the films respond optically to water under the same conditions described for VOC tests.

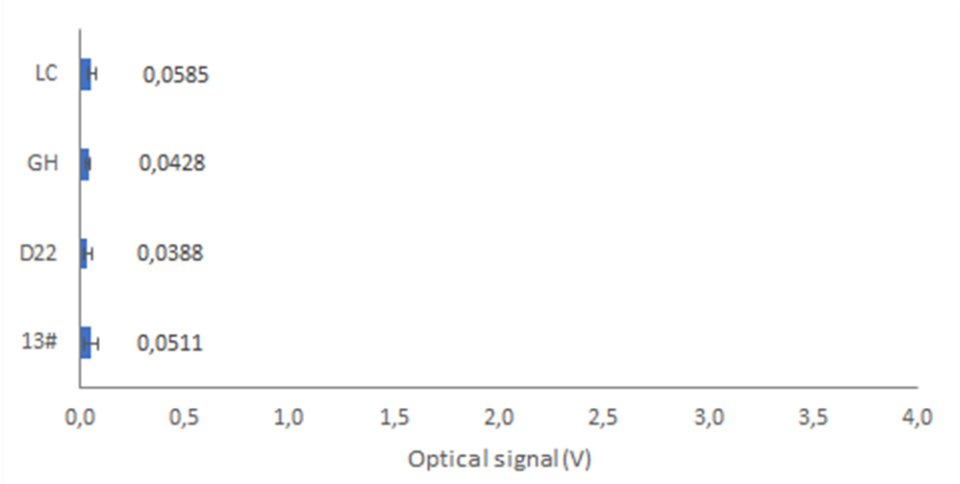

Figure 2.16 – Average amplitude range of optical responses (V) from films incorporating gelatin from bovine skin (GH), 1-docosanol (D22), suberin from 2-hour extraction of cork (13#) and liquid crystal droplets to water exposure.

Live VOC test – Polarized optical microscope VOC chamber

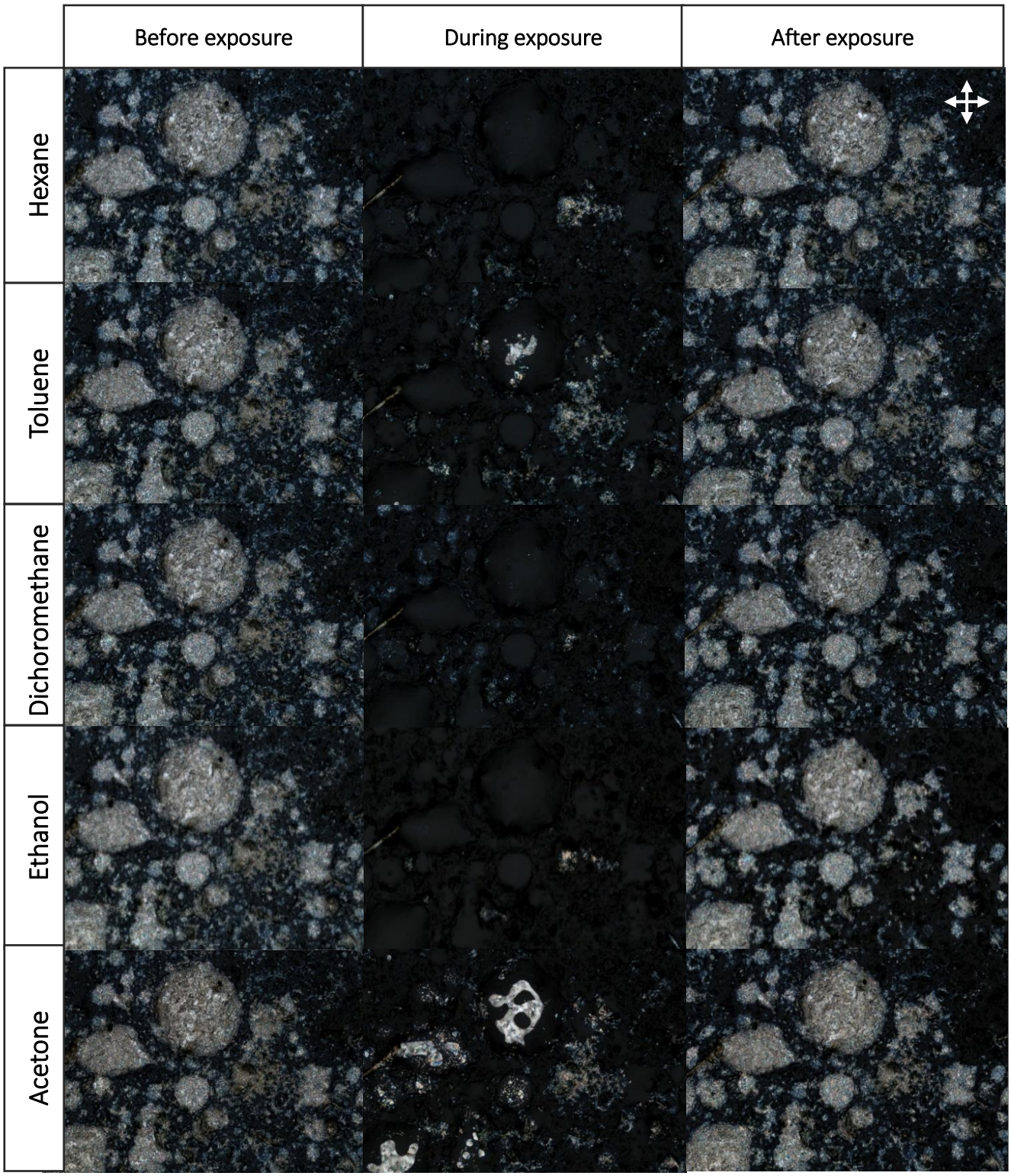

Figure 2.17 - Before, during and after exposure screenshots of live exposure of a suberin from white potato skin-based film (20P10) to hexane, toluene, dichloromethane, ethanol and acetone. The images related to the moment the volatile is present in the chamber are taken right before the effect of the volatile starts to reverse. All images were taken under crossed polarizers and at a 50x magnification.

Figure 2.17 shows screenshots of the videos recorded and reveal the before, during and after exposition to the five volatiles. Results show that, for the 20P10 film, the liquid crystal reacts when exposed to every VOC, even hexane and ethanol to which the 20P films did not respond during E-Nose tests. These results are divergent from the ones obtained from direct analysis of E-Nose signal outputs and PCA. Nevertheless, the film responds to each one of the volatiles in aa slightly different way. Acetone does not lead to a total disappearance of the liquid crystal and the film's recovery is very fast, even though it was not removed with an air pump, whilst ethanol leads to near-total inactivation of the liquid crystal and a very slow total recovery under the same conditions. Hexane, although having a lower vapour pressure then acetone, provokes a much more prominent and faster inactivation of the liquid crystal. Toluene on the other hand, has a significantly lower vapour pressure than hexane, does not inactive all the liquid crystal and the recovery is much faster than with hexane, even though the air pump was applied with the same air flow. Dichloromethane's effect on the film was very similar to that of toluene, with the different that it seems to cause a greater, although not total, inactivation of the liquid crystal, while having the highest vapour pressure of all films. No morphological modifications were perceived at the end of each test.

2.3 Discussion

The work developed in this chapter represents the first report on liquid crystal films produced in the presence of the biopolymers extracted by a non-conventional approach, namely suberin and cutin. Apart from the novelty of the approach, there are technical issues that must be considered. Firstly, the version of the E-Nose used does not allow a homogenous spread of the volatile within the detection chamber. Given the existence of 4 channels, one for each film slot, the only way to obtain comparative results for different films was to use only the channel closest to the entry of the volatile, which resulted in a 3-fold increase of the experimental procedure duration. Another limitation inherent to this device is the long plastic tube that connects the volatile chamber to the detection chamber, compromising the amount of volatile that effectively reaches the film due to condensation in the tube walls.

Secondly, the fact that there are no known optimized protocols to produce films based on the biopolymers studied, the comparison with other experimental findings is almost impossible. Also derived from lack of optimized protocols, obtaining highly homogenous films became a challenge, and while some homogeneity was attained, one cannot exclude the weight of this factor in the optical behavior of the hybrid materials. Despite these obstacles, the results obtained derived from tests held under the same conditions, meaning the error associated with the limitations aforementioned should suffer no significant deviations from tests to test.

This said, having constant exposure and recovery flows of the volatile into the E-Nose chamber (4.37L/min; 3.0L/min, respectively), heating the same volume (5mL) of all solvents in the flask and assuming the law of ideal gases can apply to all the solvents used, we can presume that higher vapour pressures lead to higher concentration of the volatile reaching the E-Nose chamber, hence, the films. This way, in case one film had no differentiated affinity towards any of the volatiles, a proportionally increasing optical response would be expected from the same led-crossed area of the film when exposed to solvents of increasing vapour pressure. This could easily explain why none of the films seem to respond to ethanol during E-Nose tests – not enough ethanol is reaching the film to provoke a significant alteration of the polymer-liquid crystal matrix. These findings seem to be further corroborated, at least relatively to the 20P formulation, by the fact that a concentrated pumping of 10mL of volatilized ethanol directly into the film leads to an immediate inactivation of the liquid crystal.

However, if increased concentration was enough to cause an optical response from the film, E-Nose tests would not have revealed that 20P films respond better to toluene than to hexane, whilst having 51.5 and 247.9 mmHg of vapour pressure under 37^{o}C, respectively. This

suggests that this correlation is not straightforward, given 20P films appear to be unresponsive to a low concentration of ethanol and responsive to a low concentration of toluene but responsive to both when highly concentrated. This observation could mean that the film is only selective when exposed to low concentrations of volatiles, postulating the existence of a threshold for the selectivity of suberin films.

Though interesting, we should consider the fact that POM only allows visualization of a small area of the film exposed, while the total area of the film is responsible for the optical response. Also, we cannot assume all films from the other formulations would behave the same when exposed to highly concentrated volumes of the volatiles. Nevertheless, the films from the 29B, 13# and TC also revealed significantly greater optical responses to toluene over hexane which prompts the idea that there could be some affinity towards toluene's molecule.

In relation to dichloromethane and acetone, higher affinity by the 29B, 13# and TC films also seems possible because, while having higher vapour pressures then toluene and hexane, they cause different responses on films with different polymer compositions. On the other hand, both films from the D22 formulation, while revealing the same tendency towards toluene, dichloromethane and acetone as the aforementioned formulations, seem to respond much weakly in amplitude. Seeing as this formulation incorporates only a suberin monomer instead of a polymerized molecule, these results might indicate that the affinity towards toluene, dichloromethane and acetone, if existent, are at least slightly related to the polymer's structure. This idea is strengthened by the fact that films from the TC formulation incorporating cutin instead of suberin also reveal a slightly lower average amplitude of response when exposed to those volatiles. Analyzing the optical responses from the GH formulation, which integrate gelatin from bovine skin instead of suberin, the assumptions made for the TC formulation can be applied.

As expected, films from the SubH formula do not respond to any of the volatiles, as there is no liquid crystal to shift the light's rotation. Moreover, liquid crystal droplets alone, while showing a small increase in optical response when exposed to dichloromethane and acetone, show an overall weaker response to all volatiles, which also suggests that the presence of a polymer gives some affinity potential to the film. Resistance to water is likely but inconclusive, given water has the lowest vapour pressure of all solvents used (47.1 mmHg) and VOC chamber tests were not performed.

Besides the apparent higher affinity of suberin-composed films to toluene, acetone and dichloromethane, all except TC films did not seem to suffer any significant changes in their

morphology after exposure to any of the VOCs. The lack resistance of cutin-based films could have something to do with the fact that the polymer might still have polysaccharides, as suggested in the discussion of Chapter 1, which results in a soluble matrix and, hence, lower resistance to the volatiles tested.

GC-MS results presented and discussed in the previous chapter could help understand why suberin-based films show the tendencies mentioned. Below are the molecular structures of the volatiles tested and two abundant monomers, 22-Hydroxydocosanoic acid in samples of suberin from cork, and Octadecanedioic acid in samples of suberin from white potato peels.

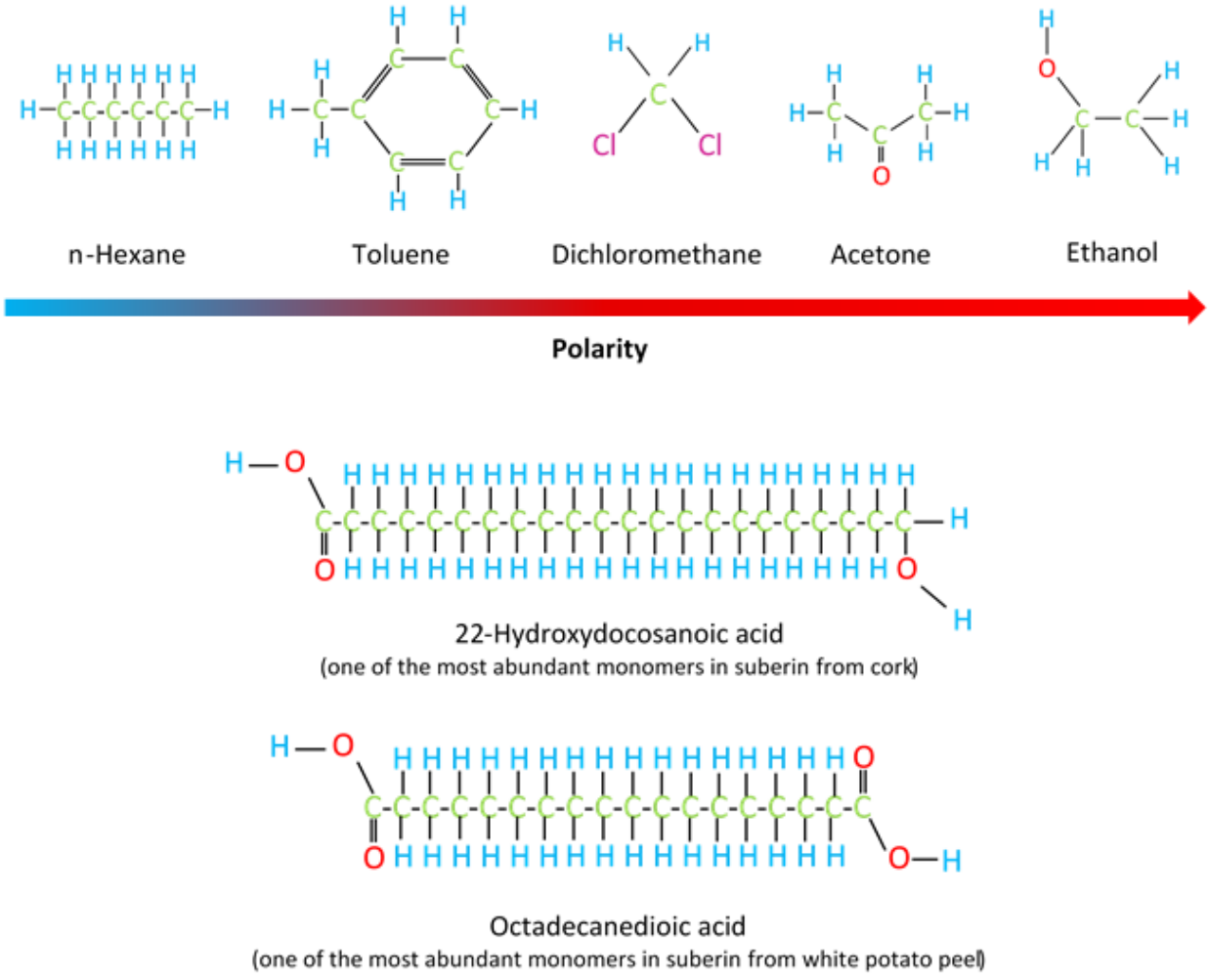

Figure 2.18 – Molecular structures of the volatiles tested, one of the most abundant monomers in suberin from cork and 1 of the most abundant monomers in suberin from white potato skin.

Assuming that the fraction of compounds identified by GC-MS is the one responsible for the biopolymers main features, 22-hydroxydocosanoic acid accounts for around 20% of relevant

components in suberin extracted from cork, and Octadecanedioic acid represents almost 35% of the relevant monomeric content. The main difference between these two monomers is the carbon-chain length (C22 and C18 respectively) and the functional groups on each end - the C22 monomer has an acidic carboxyl group in one end and a polar hydroxyl group in the other, while C18 monomer has carboxyl groups in both ends. Carboxyl groups tend to release H^+, making them acidic. On the other hand, hydroxyl groups are polar.

As Figure 2.18 reveals, hexane is a more stable molecule than toluene because the aromatic ring of toluene contains double bonds which are more reactive then single bonds, creating a small dipole moment. Alongside, the nonpolar methyl group in toluene could form hydrogen bonds with the oxygen atoms in both monomers found in suberin. This might contribute to the fact that, despite much lower VP, toluene reacts better with suberin than hexane. The high response to dichloromethane could be related to its higher polarity, very high VP and high electronegativity of the chlorine atoms, making them readily available to share electrons. As for acetone and ethanol, the fact that the latter has a VP almost 3 times lower than the first might influence the interaction with the monomers in suberin. Also, the oxygen atom in acetone can form hydrogen bonds more easily with the hydroxyl groups in the suberin monomers than ethanol. One consideration when trying to evaluate the reason behind the film's response to a certain molecule is the reversibility of the process. During E-Nose tests, all films reversed to their original state after the VOC was removed. This might indicate that the interaction was not very strong (James et al., 2005).

CONCLUSION

Overall, for each for formulation, 3 main factors were held in consideration: morphological consistency, reproducibility of optical response and different optical responses to different VOCs.

Table 2.9 – Summarized analysis of all the film formulations exposed to E-Nose tests regarding morphological consistency, reproducibility of the optical response and the capacity to respond differently to different VOCs.

	Important film attributes		
Formulations	Morphological consistency	Reproducibilty of optical response	Different responses to VOCs
Suberin 1-hour extraction from cork (29B)			
Suberin 2-hour extraction from cork (13#)			
Suberin from white potato skin (20P)			
Cutin from tomato skin (TC)			
1-Docosanol (D22)			
Gelatin from bovine skin (GH)			
Liquid crystal (LC)			
Suberin + H_2O (SubH)			

In brief, the results obtained with this work are summarized in Table 2.9 and strongly suggest that the biopolymers suberin and cutin have the potential to enhance the selectivity of gas sensing materials. Moreover, the findings reveal that suberin is an excellent choice as stabilizing agent, given the lack of morphological changes detected by POM after exposure to different volatiles. One straightforward conclusion is that the liquid crystal does not need to be arranged in a radial orientation to be able to provide a change in optical response. This could be a big advantage, seeing as the previously used films consisted in a specific mixture of 5CB with [BMIM][DCA] ionic liquid to provide the optimum conditions for the formation of radial-oriented LC. The prospect of not needing ionic liquid is advantageous in many ways, given it allows cost and time reductions, while sparing the usage of complex or expensive equipment, such as a spin-coater to produce the films. No conclusions can be drawn for the behavior of the polymers in the presence of the volatiles studied, but nonetheless, one can assume that it is the result of many factors, such as polarity of the molecules involved, volatile concentration, vapour pressure of the compounds, the existence of nanostructures within the polymer matrix, the interaction between the polymers and the liquid crystal and the way the polymers were extracted from the source. Despite this, the fact that suberin-based films constantly revealed higher responses to toluene, dichloromethane and acetone, over cutin-based, monomer-based and gelatin-based films strongly corroborates the concept that different biopolymers can convey selectivity to different volatiles.

FUTURE WORK

When we think about gas sensors, it is easy to point out the characteristics it should have: robustness, selectivity, sensitivity and reliability. Other than that, cost-effectiveness, easy manipulation and practicability are also in sight. Despite the many advances and milestones achieved in the area of electronic noses for medical and industrial applications, new discoveries are always just around the corner. The motivation behind this thesis was to test new biopolymers as gas sensors and try to answer the question to whether they could be used to detect volatile organic compounds. The results obtained throughout the work seems to indicate that they can, indeed, enhance the selectivity of sensing materials and thus contribute to more effective arrays of gas sensors. Nevertheless, this work is just the basis for a deeper attempt at discovering the full potential of these biopolymers for such application. A more extensive analysis of the composition of the biopolymers, such as X-ray photoelectron spectroscopy (XPS), would certainly help understand the behavior of the formulations produced during the E-Nose tests. Also, testing all these volatiles under the live VOC-test chamber is crucial to understand what really happens to the liquid crystal during exposure.

Regarding the pre-treatment of the biomass before extraction of the polymer, there is still the need to optimize the enzymatic hydrolysis, which could be achieved by using more specific enzymes. For that, a closer analysis of the polysaccharidic composition of the biomass is needed beforehand. The cholinium hexanoate-mediated extraction also needs optimization due to fluctuating polymer yields. Moreover, the extraction of polyesters from other sources, such as Rocha pear skin and corn silk, initiated but not completed during this work, could also provide valuable information and products of uttermost importance to the fabrication of materials with novel characteristics and innovative applications. For the analysis of the polymer yield it is important to consider the polymer content in the source and, although research was made on that matter, the yields presented did not consider those theoretical contents and, as such, it should be something to consider in the future.

Concerning the production of films, the optimization of the protocol used is crucial for the homogeneity and reproducibility of the signals produced by VOC exposure in the E-Nose. For that, a suitability of the protocol for each type of polyester is needed, given the great structural diversity. Furthermore, it would be interesting to produce films using the most abundant monomers in to each polyester, expose them to the VOCs that lead to better E-Nose responses and compare with the partially intact polyester. This would provide information regarding the usefulness of the extraction method applied for the specific application of suberin and cutin as part of stimuli responsive hybrid materials.

REFERENCES

Aernecke, M. J., & Walt, D. R. (2009). Optical-fiber arrays for vapor sensing. *Sensors and Actuators, B: Chemical*, *142*(2), 464–469. https://doi.org/10.1016/j.snb.2009.06.054

Al-Wandawi, H., Abdul-Rahman, M., & Al-Shaikhly, K. (1985). Tomato Processing Wastes as Essential Raw Materials Source. *Journal of Agricultural and Food Chemistry*, *33*(5), 804–807. https://doi.org/10.1021/jf00065a009

Benítez, J. J., Matas, A. J., & Heredia, A. (2004). Molecular characterization of the plant biopolyester cutin by AFM and spectroscopic techniques. *Journal of Structural Biology*, *147*(2), 179–184. https://doi.org/10.1016/j.jsb.2004.03.006

Bo, L., Xiao, C., Hualin, C., Mohammad, M., Xiangguang, T., Luqi, T., Yang, Y., Tianling, R. (2016). Surface Acoustic Wave Devices for Sensor Applications. *Journal of Semiconductors*, *37*(2), 9.

Carlton, R. J., Hunter, J. T., Miller, D. S., Abbasi, R., Mushenheim, P. C., Tan, L. N., & Abbott, N. L. (2013). Chemical and biological sensing using liquid crystals. *Liquid Crystals Reviews*, *1*(1), 29–51. https://doi.org/10.1080/21680396.2013.769310

Cifarelli, A., Cigognini, I., Bolzoni, L., & Montanari, A. (2014). Cutin isolated from tomato processing by-products : extraction methods and characterization ., 1–20.

Cordeiro, N., Belgacem, M. N., Silvestre, A. J. D., Pascoal Neto, C., & Gandini, A. (1998). Cork suberin as a new source of chemicals. 1. Isolation and chemical characterization of its composition. *International Journal of Biological Macromolecules*, *22*(2), 71–80. https://doi.org/10.1016/S0141-8130(97)00090-1

Elbadrawy, E., & Sello, A. (2016). Evaluation of nutritional value and antioxidant activity of tomato peel extracts. *Arabian Journal of Chemistry*, *9*, S1010–S1018. https://doi.org/10.1016/j.arabjc.2011.11.011

Ferguson, L. R., & Harris, P. J. (1998). Suberized plant cell walls suppress formation of heterocyclic amine-induced aberrant crypts in a rat model. *Chemico-Biological Interactions*, *114*(3), 191–209. https://doi.org/10.1016/S0009-2797(98)00054-4

Ferreira, R., Garcia, H., Sousa, A. F., Petkovic, M., Lamosa, P., Freire, C. S. R., … Liang, D. T. (2012). Suberin isolation from cork using ionic liquids: characterisation of ensuing products. *New Journal of Chemistry*, *36*(10), 2014. https://doi.org/10.1039/c2nj40433h

Ferreira, R., Garcia, H., Sousa, A. F., Freire, C. S. R., Silvestre, A. J. D., Rebelo, L. P. N., & Silva Pereira, C. (2013). Isolation of suberin from birch outer bark and cork using ionic liquids: A new source of macromonomers. *Industrial Crops and Products*, *44*, 520–527. https://doi.org/10.1016/j.indcrop.2012.10.002

Ferreira, R., Garcia, H., Sousa, A. F., Guerreiro, M., Duarte, F. J. S., Freire, C. S. R., Saramago, B. (2014). Unveiling the dual role of the cholinium hexanoate ionic liquid as solvent and catalyst in suberin depolymerisation. *RSC Adv.*, *4*(6), 2993–3002. https://doi.org/10.1039/C3RA45910A

Gandini, A., Pascoal Neto, C., & Silvestre, A. J. D. (2006). Suberin: A promising renewable resource for novel macromolecular materials. *Progress in Polymer Science (Oxford)*, *31*(10), 878–892. https://doi.org/10.1016/j.progpolymsci.2006.07.004

Garcia, H., Ferreira, R., Martins, C., Sousa, A. F., Freire, C. S. R., Silvestre, A. J. D., … Silva Pereira, C. (2014). Ex situ reconstitution of the plant biopolyester suberin as a film. *Biomacromolecules*, *15*(5), 1806–1813. https://doi.org/10.1021/bm500201s

Garcia, H., Ferreira, R., Petkovic, M., Ferguson, J. L., Leitão, M. C., Gunaratne, H. Q. N., … DiFiore, D. (2010). Dissolution of cork biopolymers in biocompatible ionic liquids. *Green Chemistry*, *12*(3), 367. https://doi.org/10.1039/b922553f

Gardner, J. W., & Bartlett, P. N. (1994). A brief history of electronic noses*. *Sensors and Actuators B*, *19*, 18–19. https://doi.org/10.1016/0925-4005(94)87085-3

Graça, J., & Pereira, H. (2000). Suberin structure in potato periderm: Glycerol, long-chain monomers, and glyceryl and feruloyl dimers. *Journal of Agricultural and Food Chemistry*, *48*(11), 5476–5483. https://doi.org/10.1021/jf0006123

Heinämäki, J., Pirttimaa, M. M., Alakurtti, S., Pitkänen, H. P., Kanerva, H., Hulkko, J., Kogermann, K. (2017). Suberin Fatty Acids from Outer Birch Bark: Isolation and Physical Material Characterization. *Journal of Natural Products*, *80*(4), 916–924. https://doi.org/10.1021/acs.jnatprod.6b00771

Hussain, A., Pina, A. S., & Roque, A. C. A. (2009). Bio-recognition and detection using liquid crystals. *Biosensors and Bioelectronics*, *25*(1), 1–8. https://doi.org/10.1016/j.bios.2009.04.038

Hussain, A., Semeano, A. T. S., Palma, S. I. C. J., Pina, A. S., Almeida, J., Medrado, B. F., Roque, A. C. A. (2017). Tunable Gas Sensing Gels by Cooperative Assembly. *Advanced Functional Materials*, *27*(27), 1–9. https://doi.org/10.1002/adfm.201700803

Isaacson, T., Kosma, D. K., Matas, A. J., Buda, G. J., He, Y., Yu, B., Rose, J. K. C. (2009). Cutin deficiency in the tomato fruit cuticle consistently affects resistance to microbial infection and biomechanical properties, but not transpirational water loss. *Plant Journal*, *60*(2), 363–377. https://doi.org/10.1111/j.1365-313X.2009.03969.x

James, D., Scott, S. M., Ali, Z., & O'Hare, W. T. (2005). Chemical sensors for electronic nose systems. *Microchimica Acta*, *149*(1–2), 1–17. https://doi.org/10.1007/s00604-004-0291-6

Kolattukudy, P. E., & Agrawal, V. P. (1974). Structure and composition of aliphatic constituents of potato tuber skin (suberin). *Lipids*, *9*(9), 682–691. https://doi.org/10.1007/BF02532176

Lara, I., Belge, B., & Goulao, L. F. (2015). A focus on the biosynthesis and composition of cuticle in fruits. *Journal of Agricultural and Food Chemistry*, *63*(16), 4005–4019. https://doi.org/10.1021/acs.jafc.5b00013

Li-Beisson, Y. (2011). Cutin and Suberin. *Encyclopedia of Life Sciences*, (March), 1–9. https://doi.org/DOI: 10.1002/9780470015902.a0001920.pub2

Lopes, M. H., Gil, A. M., Silvestre, A. J. D., & Neto, C. P. (2000). Composition of suberin extracted upon gradual alkaline methanolysis of Quercus suber L. Cork. *Journal of Agricultural and Food Chemistry*, *48*(2), 383–391. https://doi.org/10.1021/jf9909398

Martin, L. B. B., & Rose, J. K. C. (2014). There's more than one way to skin a fruit: Formation and functions of fruit cuticles. *Journal of Experimental Botany*, *65*(16), 4639–4651. https://doi.org/10.1093/jxb/eru301

Mattinen, M. L., Filpponen, I., Järvinen, R., Li, B., Kallio, H., Lektinen, P., & Argyropoulos, D. (2009). Structure of the polyphenolic component of suberin isolated from potato (Solanum tuberosum war. Nikola). *Journal of Agricultural and Food Chemistry*, *57*(20), 9747–9753. https://doi.org/10.1021/jf9020834

Meixner, H., & Lampe, U. (1996). Metal oxide sensors. *Sensors and Actuators B: Chemical*, *33*(1–3), 198–202. https://doi.org/10.1016/0925-4005(96)80098-0

Nielsen, S. S., Science, F., & Series, T. (2010). Food Analysis Laboratory Manual, 47–53. https://doi.org/10.1007/978-1-4419-1463-7

Pereira, H. (1988). Chemical composition and variability of cork from Quercus suber L. *Wood Science and Technology*, *22*(3), 211–218. https://doi.org/10.1007/BF00386015

Pereira, H. (2015). The Rationale behind Cork Properties: A Review of Structure and Chemistry. *Biorresources*, *10*(2005), 6207–6229. https://doi.org/10.15376/biores.10.3.Pereira

Petkovic, M., Ferguson, J. L., Gunaratne, H. Q. N., Ferreira, R., Leitão, M. C., Seddon, K. R., … Pereira, C. S. (2010). Novel biocompatible cholinium-based ionic liquids—toxicity and biodegradability. *Green Chemistry*, *12*(4), 643. https://doi.org/10.1039/b922247b

Pinto, P. C. R. O., Sousa, A. F., Silvestre, A. J. D., Neto, C. P., Gandini, A., Eckerman, C., & Holmbom, B. (2009). Quercus suber and Betula pendula outer barks as renewable sources of oleochemicals: A comparative study. *Industrial Crops and Products*, *29*(1), 126–132. https://doi.org/10.1016/j.indcrop.2008.04.015

Poziomek, E. J., Novak, T. J., & Mackay, R. a. (1974). Use of Liquid Crystals as Vapor Detectors. *Molecular Crystals and Liquid Crystals*, *27*(1–2), 175–185. https://doi.org/10.1080/15421407408083128

Schieber, A., & Saldaña, M. (2009). Potato Peels : A Source of Nutritionally and Pharmacologically Interesting Compounds – A Review. *Food*, *3*, 23–29. Retrieved from http://www.globalsciencebooks.info/JournalsSup/images/0906/FOOD_3(SI2)23-29o.pdf

Sidibé, A., Simao-Beaunoir, A.-M., Lerat, S., Giroux, L., Toussaint, V., & Beaulieu, C. (2016). Proteome Analyses of Soil Bacteria Grown in the Presence of Potato Suberin, a Recalcitrant Biopolymer. *Microbes and Environments*, *31*(4), 418–426. https://doi.org/10.1264/jsme2.ME15195

Silva, L. T. (2015). Evaluating the potential of yeast strains to produce added value products for the food and / or pharmaceutical industries.

Walton, T. J., & Kolattukudy, P. E. (1972). Determination of the structures of cutin monomers by a novel depolymerization procedure and combined gas chromatography and mass spectrometry. *Biochemistry*, *11*(10), 1885–1897. https://doi.org/10.1021/bi00760a025

APPENDIX

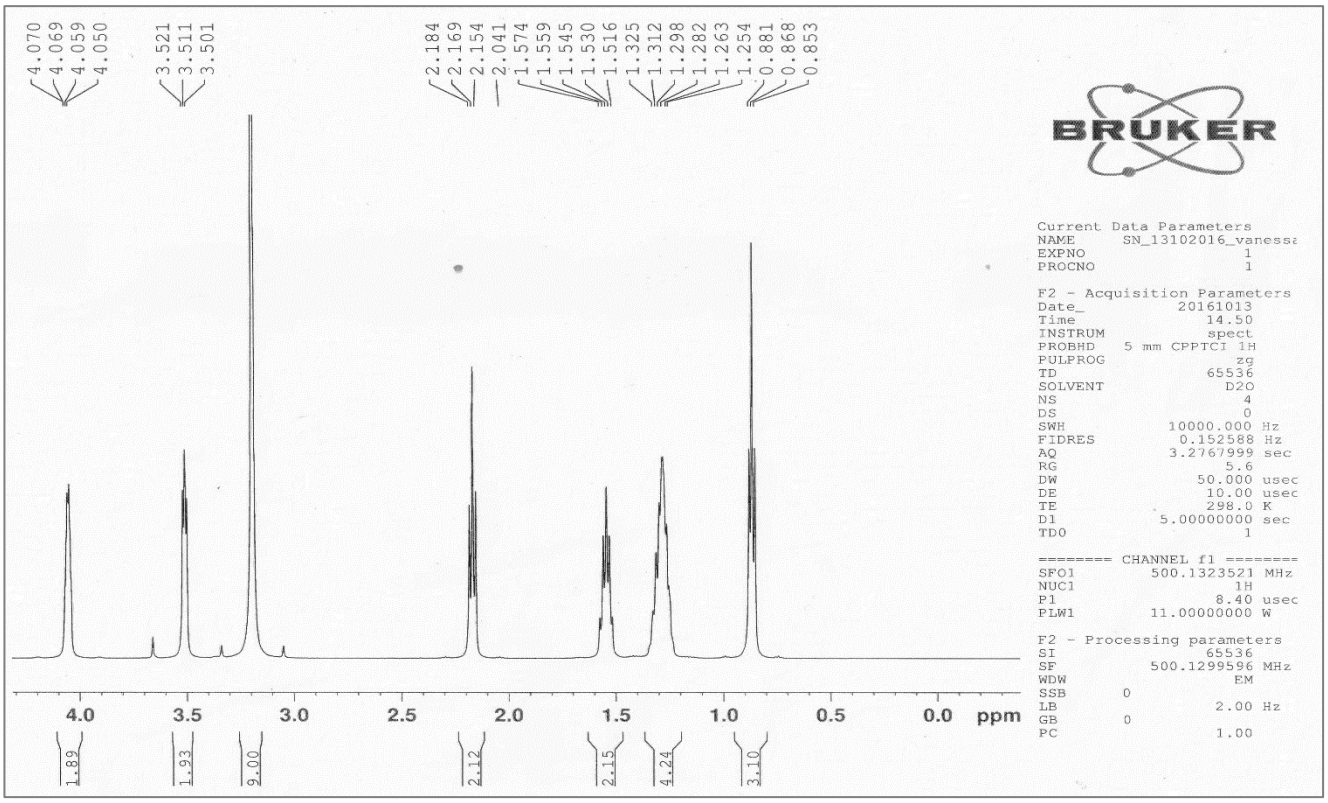

Figure 1.21 - ^{1}H-NMR analysis performed on Cholinium Hexanoate after production and before used for polymer extraction.

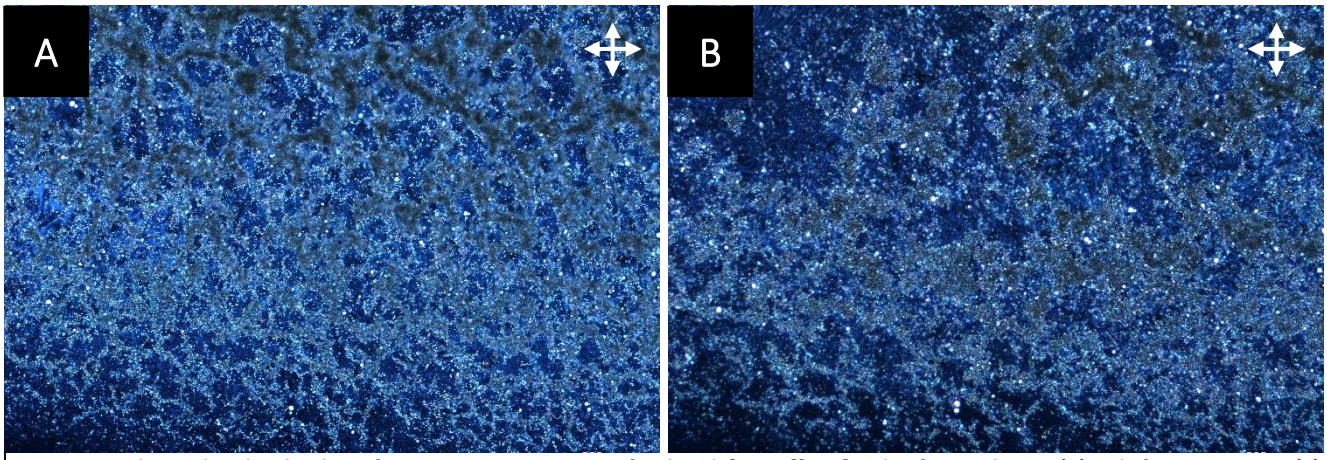

Figure 2.19 - Film produced with suberin from 30-minute extraction of cork with [BMIM][DCA], right after production (A) and after E-Nose tests (B). 50x magnification.

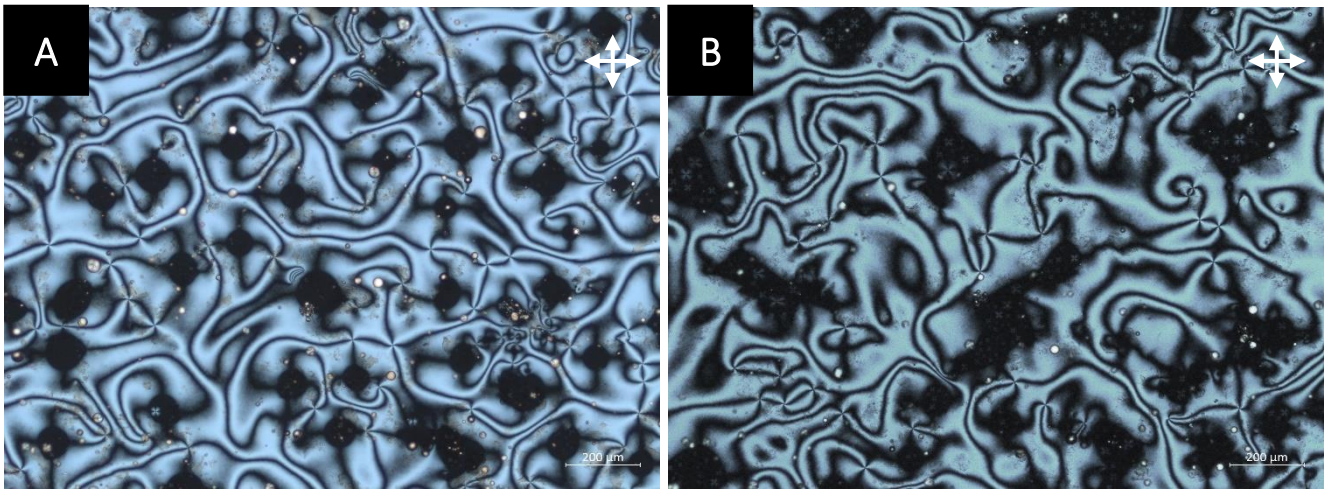

Figure 2.20 - Film produced with suberin from 30-minute extraction of cork and cholinium hexanoate, right after production (A) and after 25 days (B). 50x magnification.

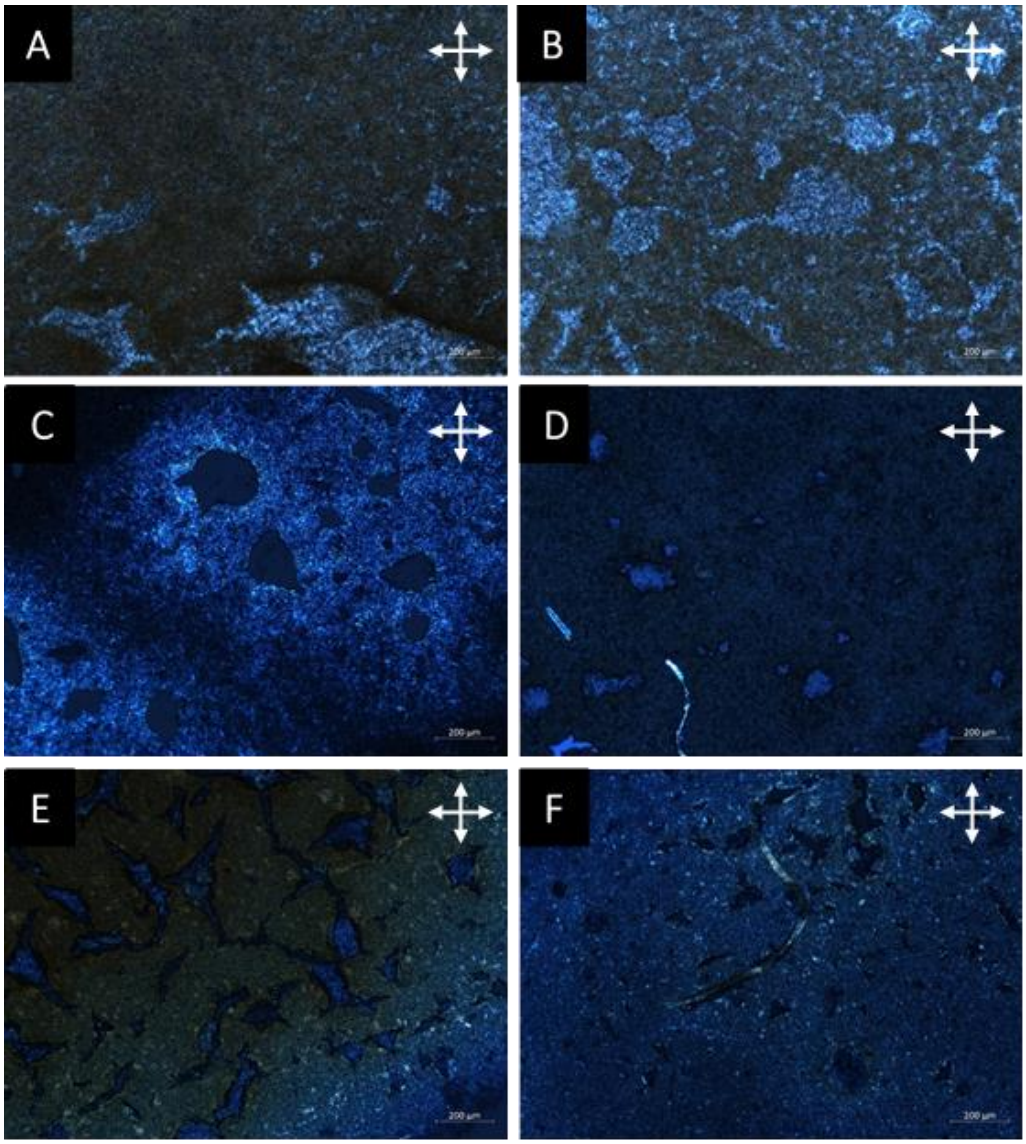

Figure 2.21 - POM images of the films produced with the 29A formulation. 50x magnification. A: 29A1; B: 29A2; C:29A3; D:29A4.; E: 29A5; F: 29A6.

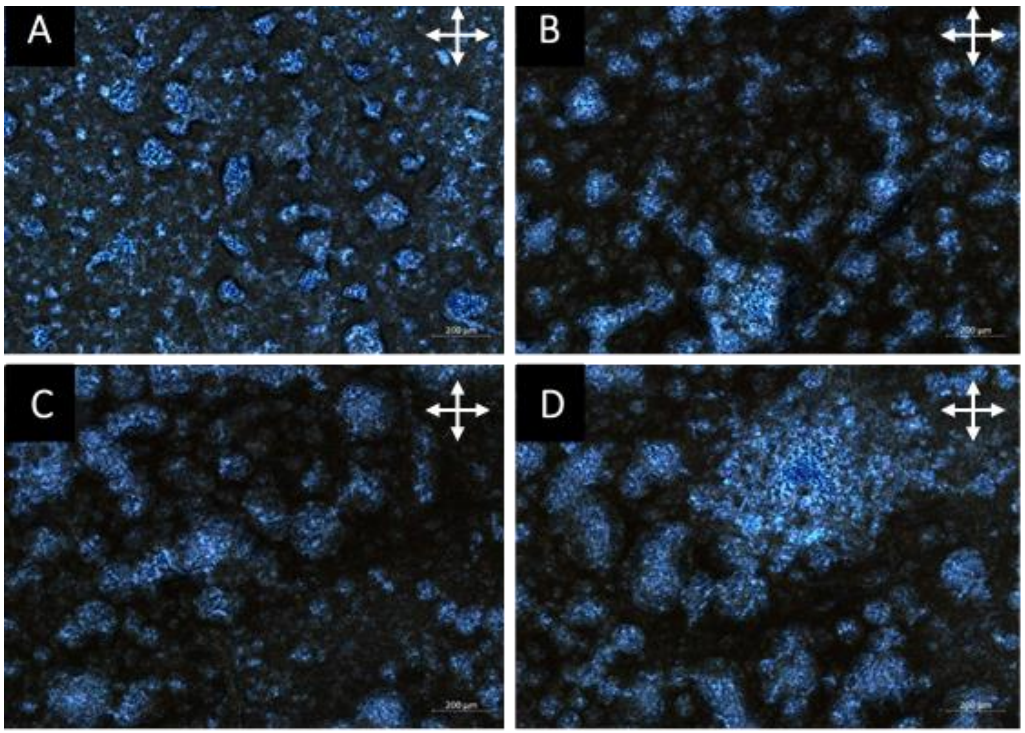

Figure 2.22 - POM images of the films produced with the 29B formulation. 50x magnification. A: 29B1; B: 29B2; C:29B3; D:29B4.

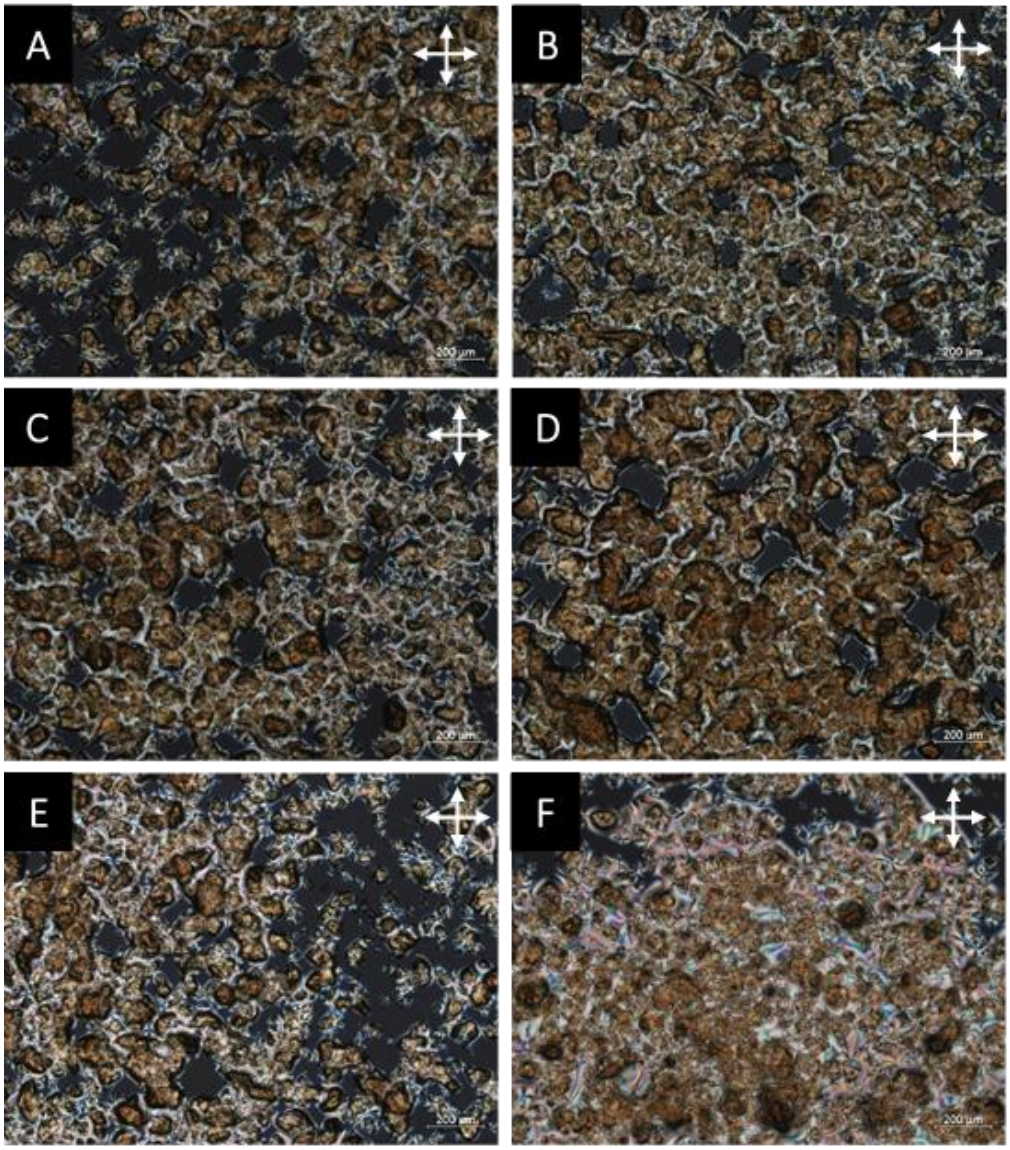

Figure 2.23 - POM images of the films produced with the 13# formulation. 50x magnification. A: 13A; B: 13B; C:13C; D:13D; E: 13E; F: 13F.

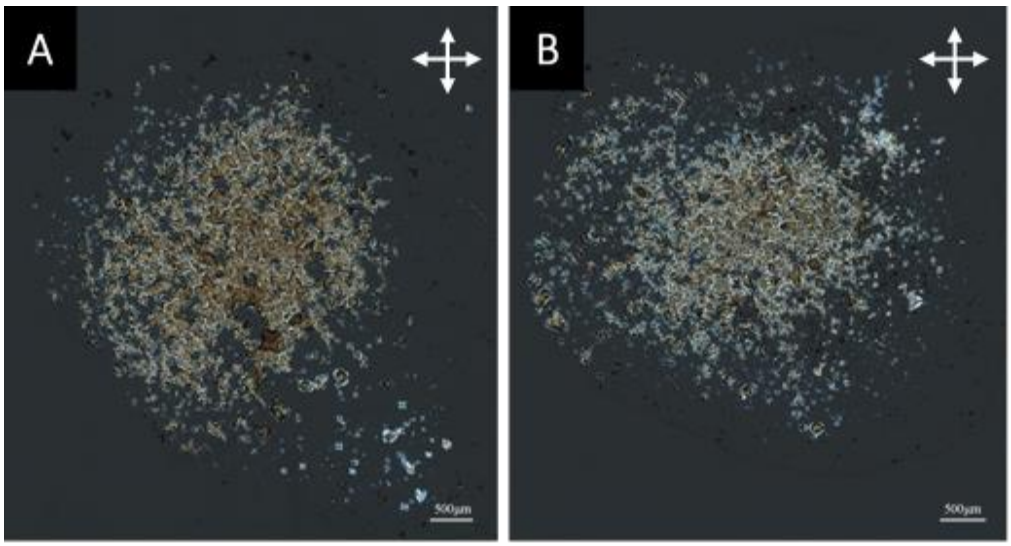

Figure 2.24 - POM Tile images of 13B and 13D 2 days after production (A and B, respectively, before E-Nose tests. 50x magnification.

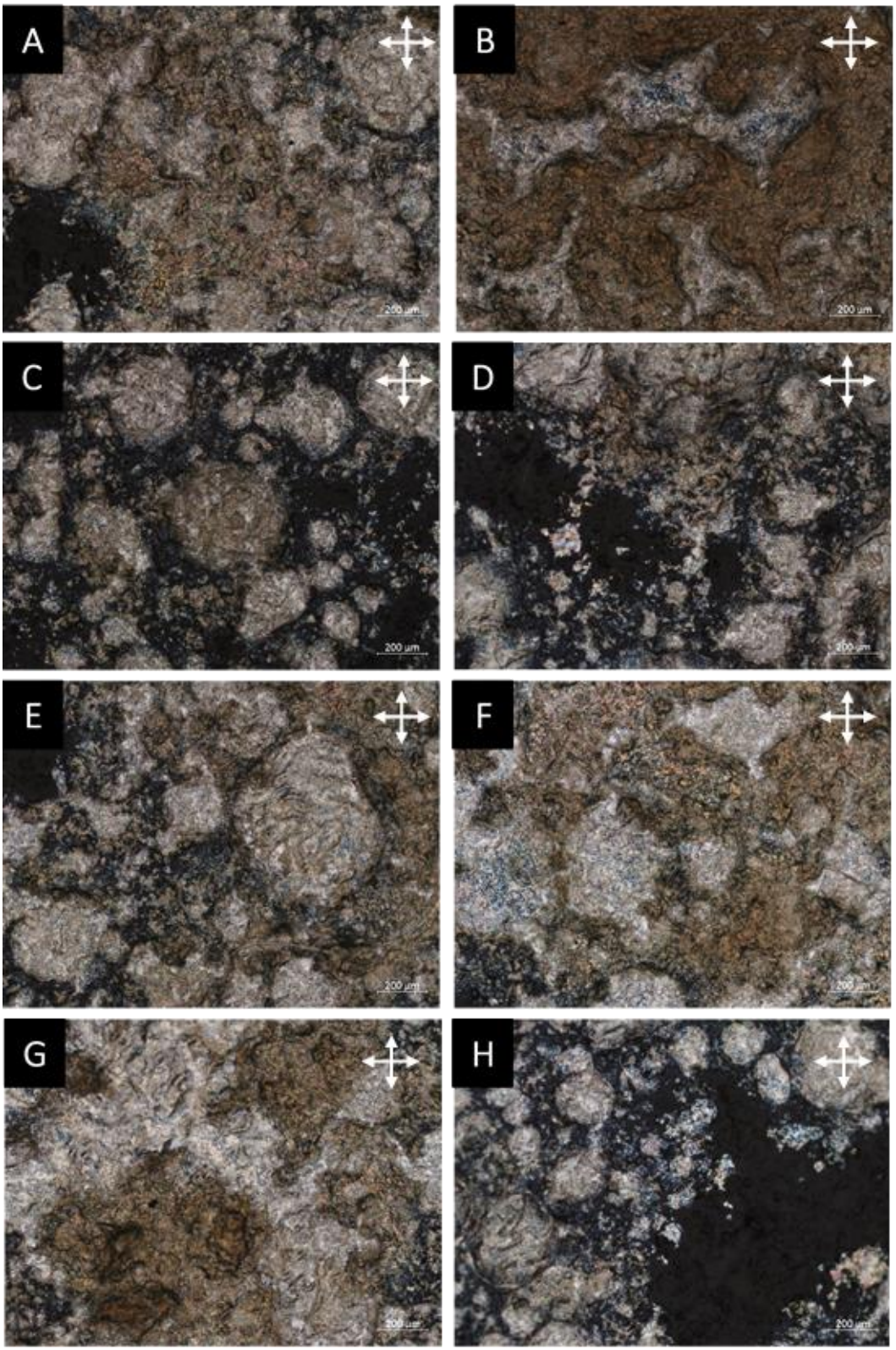

Figure 2.25- POM images of the films produced with the 20P formulation. 50x magnification. A: 20P1; B: 20P2; C:20P3; D:20P4; E: 20P5; F: 20P6; E: 20P7; F: 20P8.

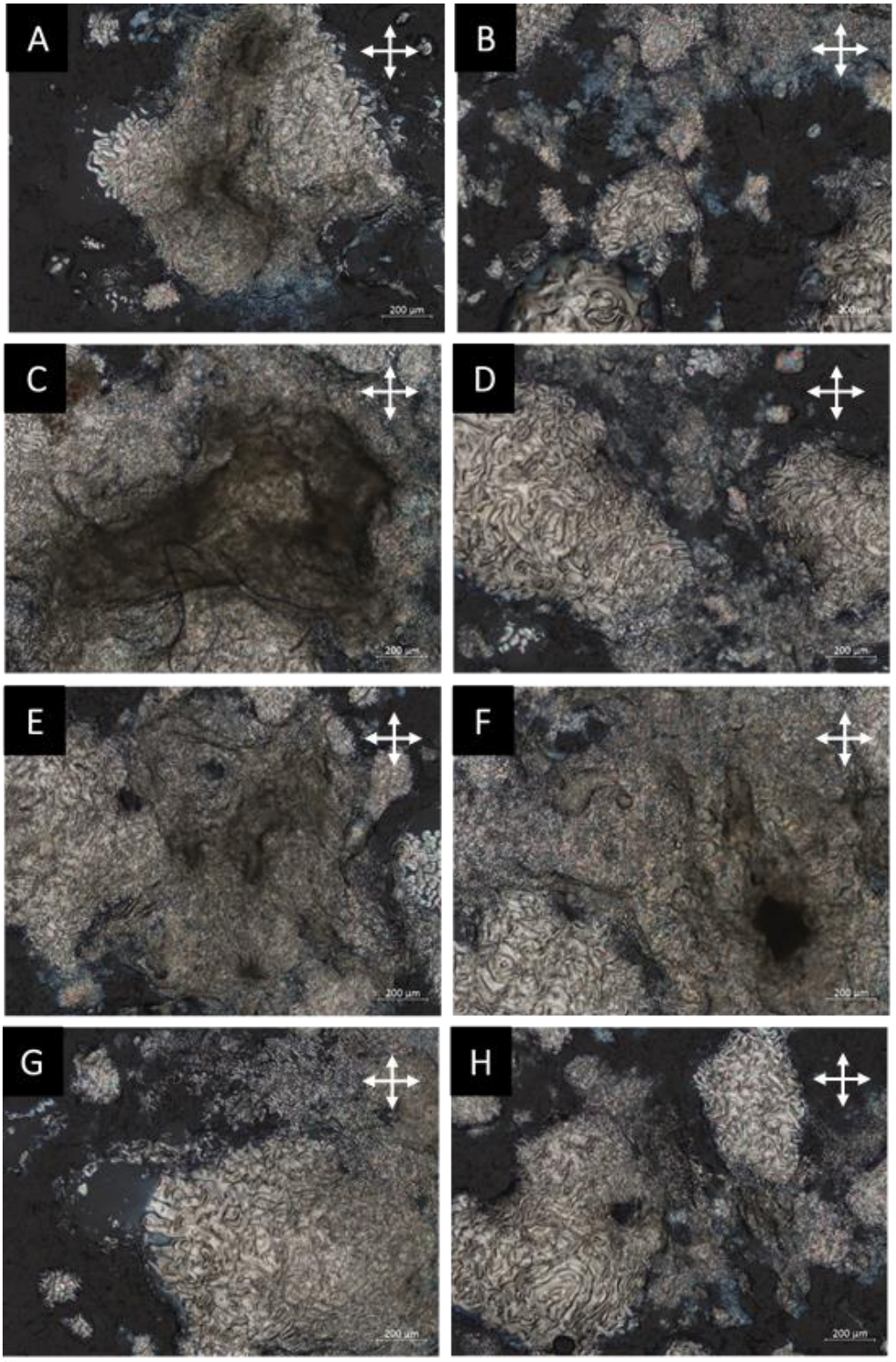

Figure 2.26 - POM images of the films produced with the TC formulation. 50x magnification. A: TC1; B: TC2; C:TC3; D:TC4; E: TC5; F: TC6; G: TC7; H:TC8.

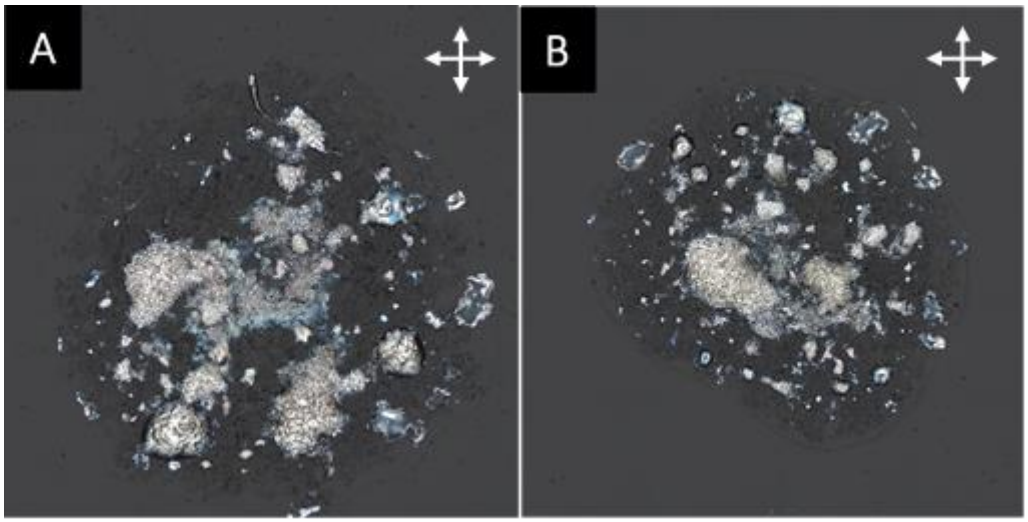

Figure 2.27 - POM Tile images of TC2 and TC4 after production (A and B, respectively, before E-Nose tests. 50x magnification.

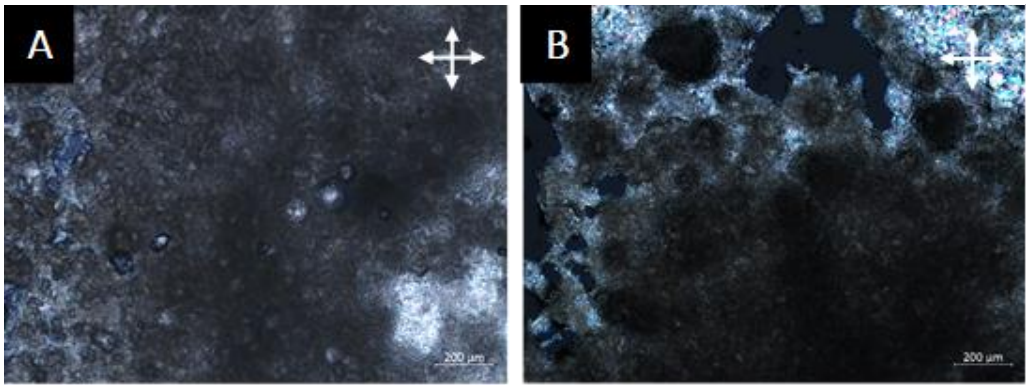

Figure 2.28 - POM images of the films produced with the D22 formulation. 50x magnification. A: D22B; B: D22C.

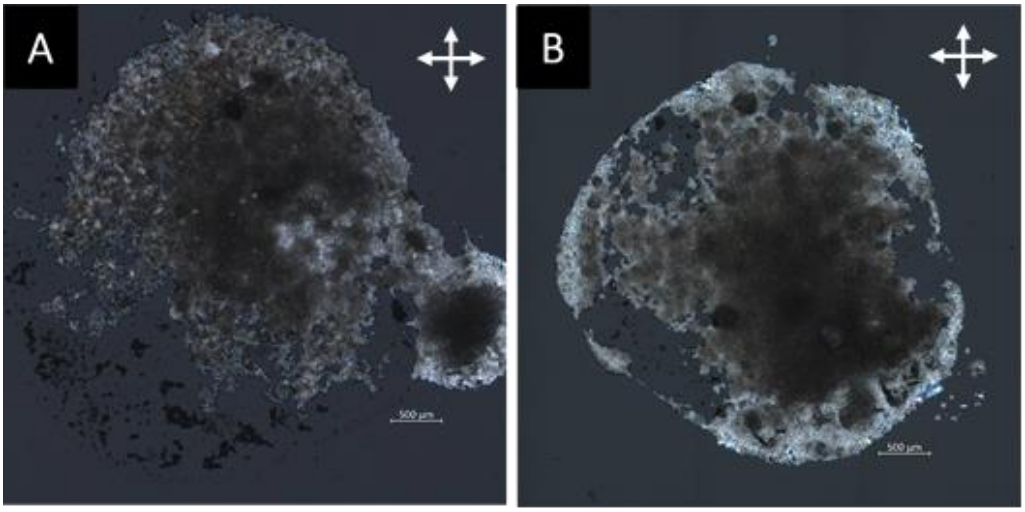

Figure 2.29 - POM Tile images of D22B and D22C after production (A and B, respectively, before E-Nose tests. 50x magnification.

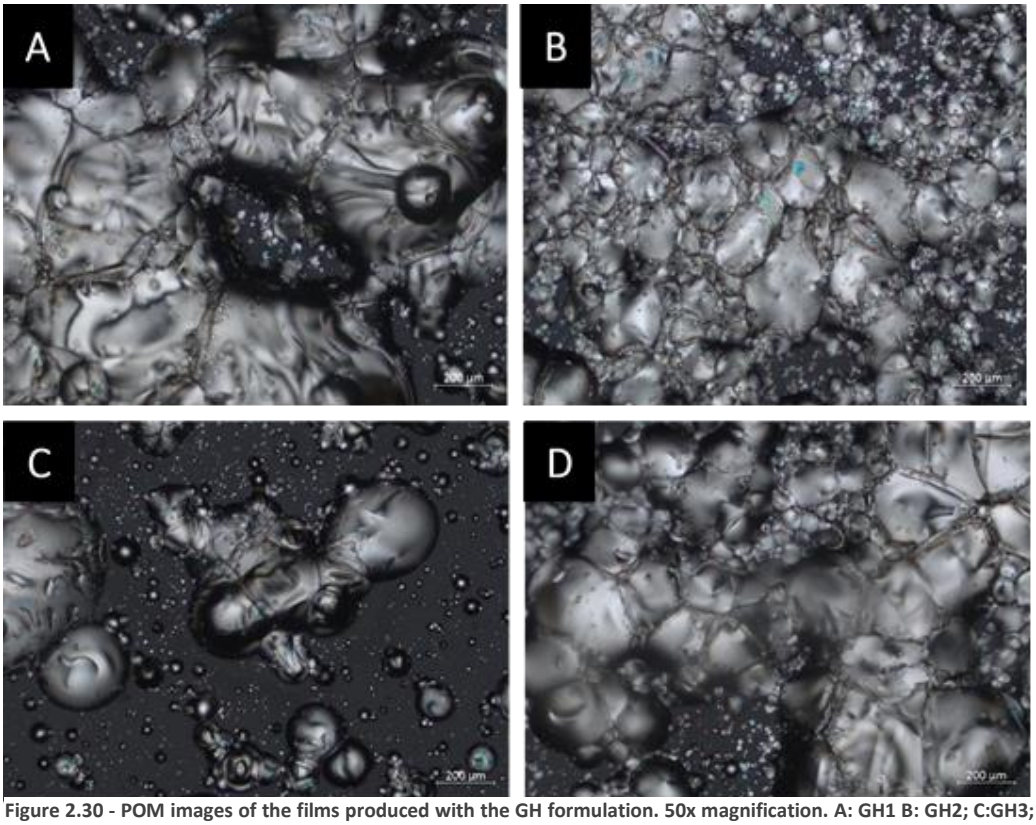

Figure 2.30 - POM images of the films produced with the GH formulation. 50x magnification. A: GH1 B: GH2; C:GH3; D:GH4.

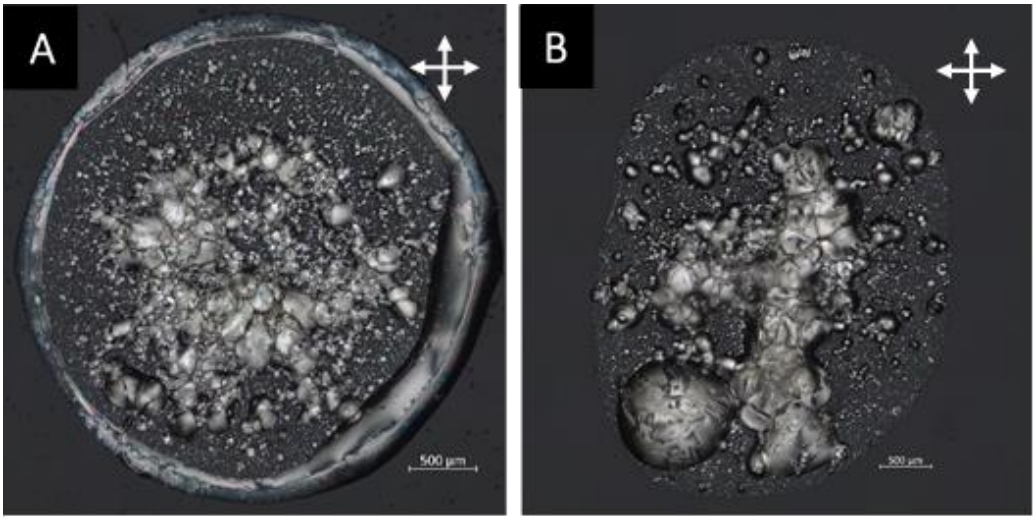

Figure 2.31 - POM Tile images of GH2 and GH4 after production (A and B, respectively, before E-Nose tests. 50x magnification.

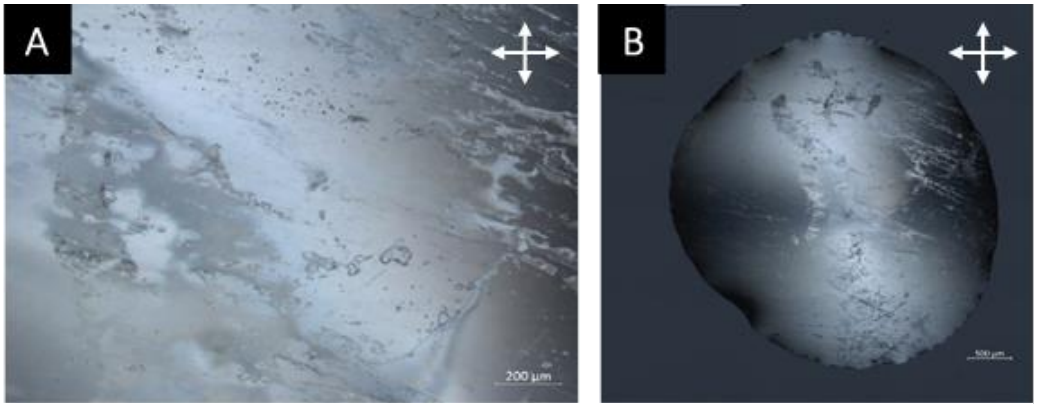

Figure 2.32 - POM images of LC1 in closeup view and tile (A and B, respectively) after production and before E-Nose testing. 50x magnification.

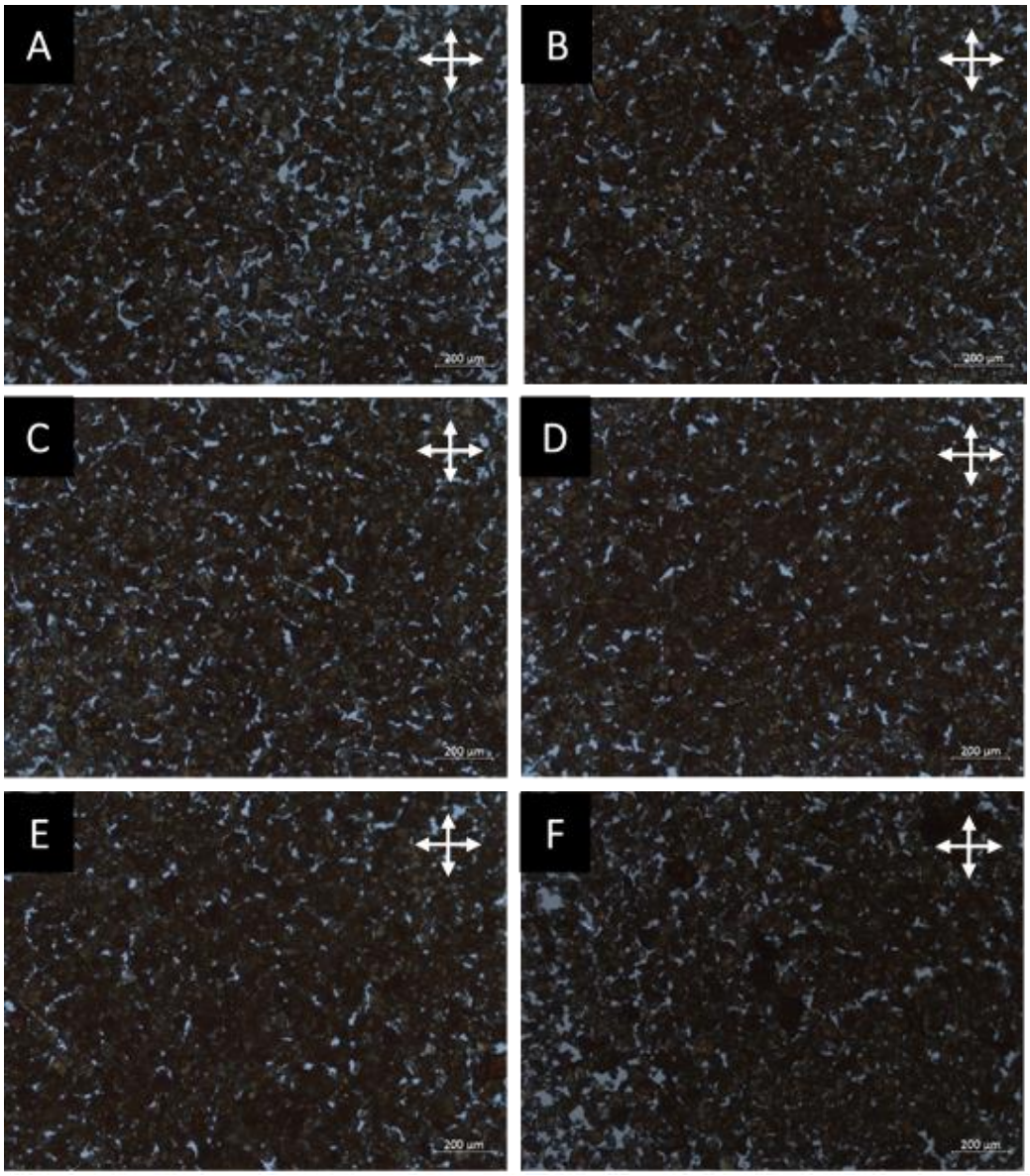

Figure 2.33 - POM images of the films produced with the SubH formulation. 50x magnification. A: SubH1; B: SubH2; C: SubH3; D: SubH4; E: SubH5; F: SubH6.

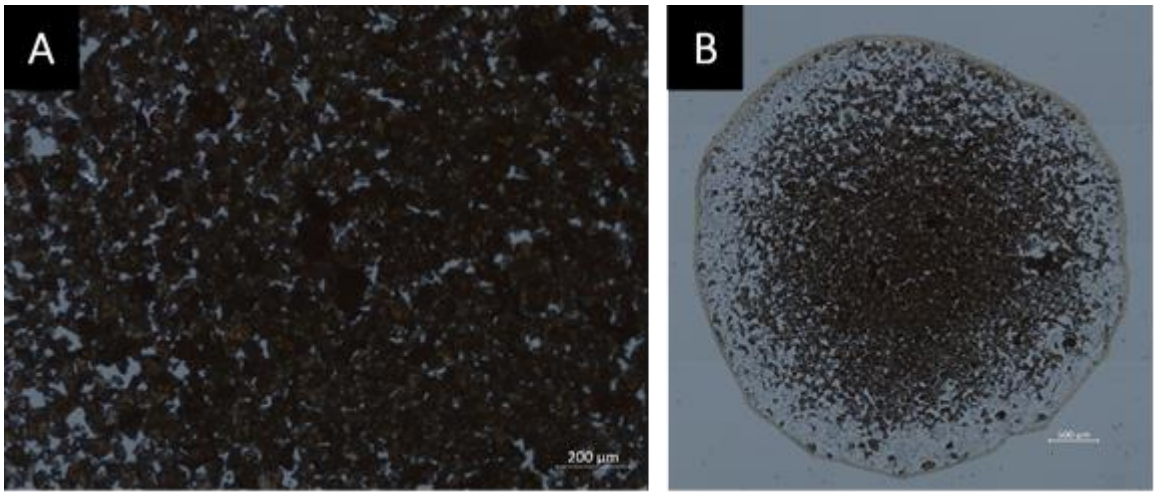

Figure 2.34 - POM images of SubH6 in closeup view and tile (A and B, respectively) after production and before E-Nose testing. 50x magnification.

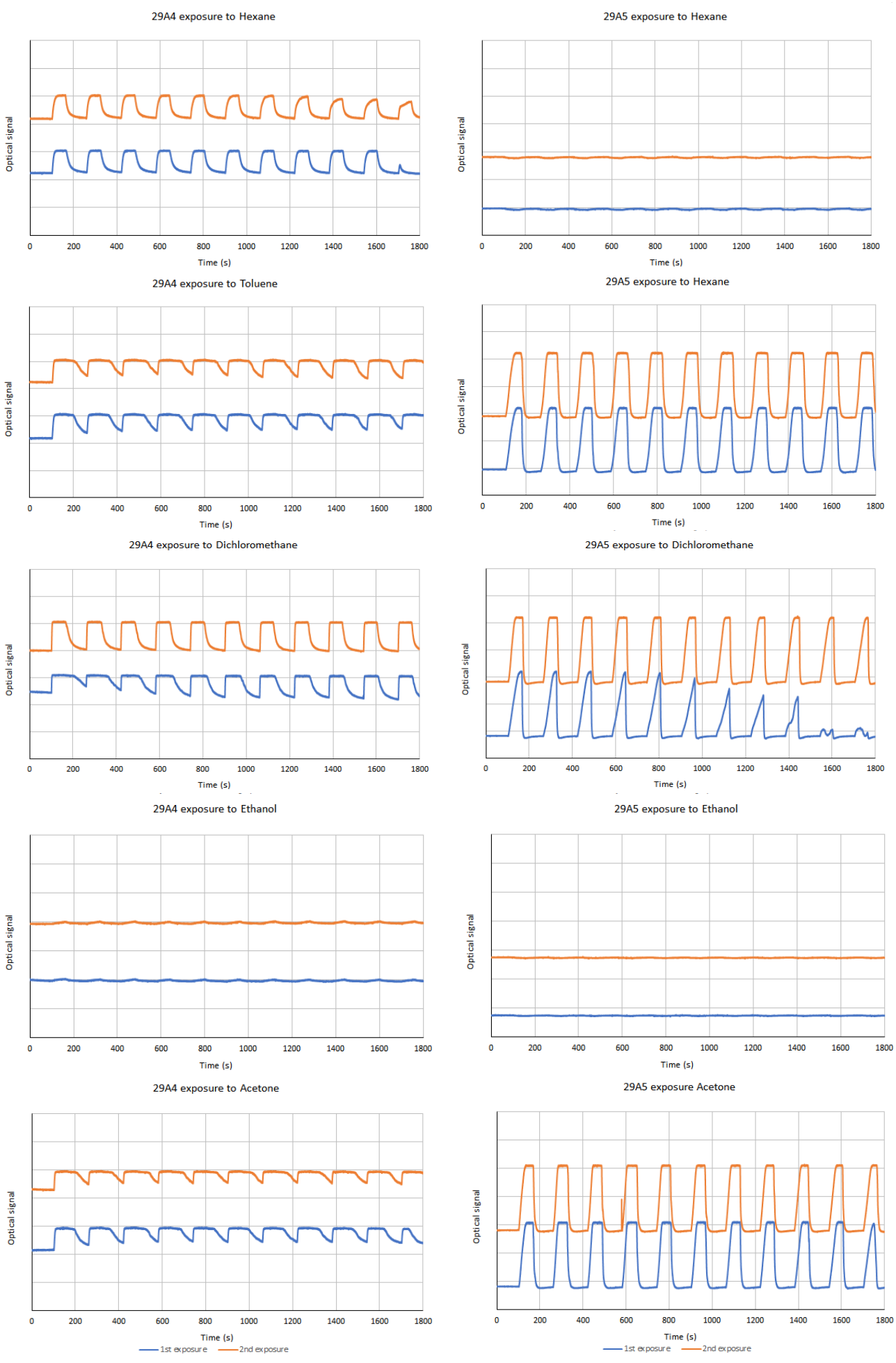

Figure 2.35 – E-Nose tests on films with suberin from 30-minute extraction of cork.

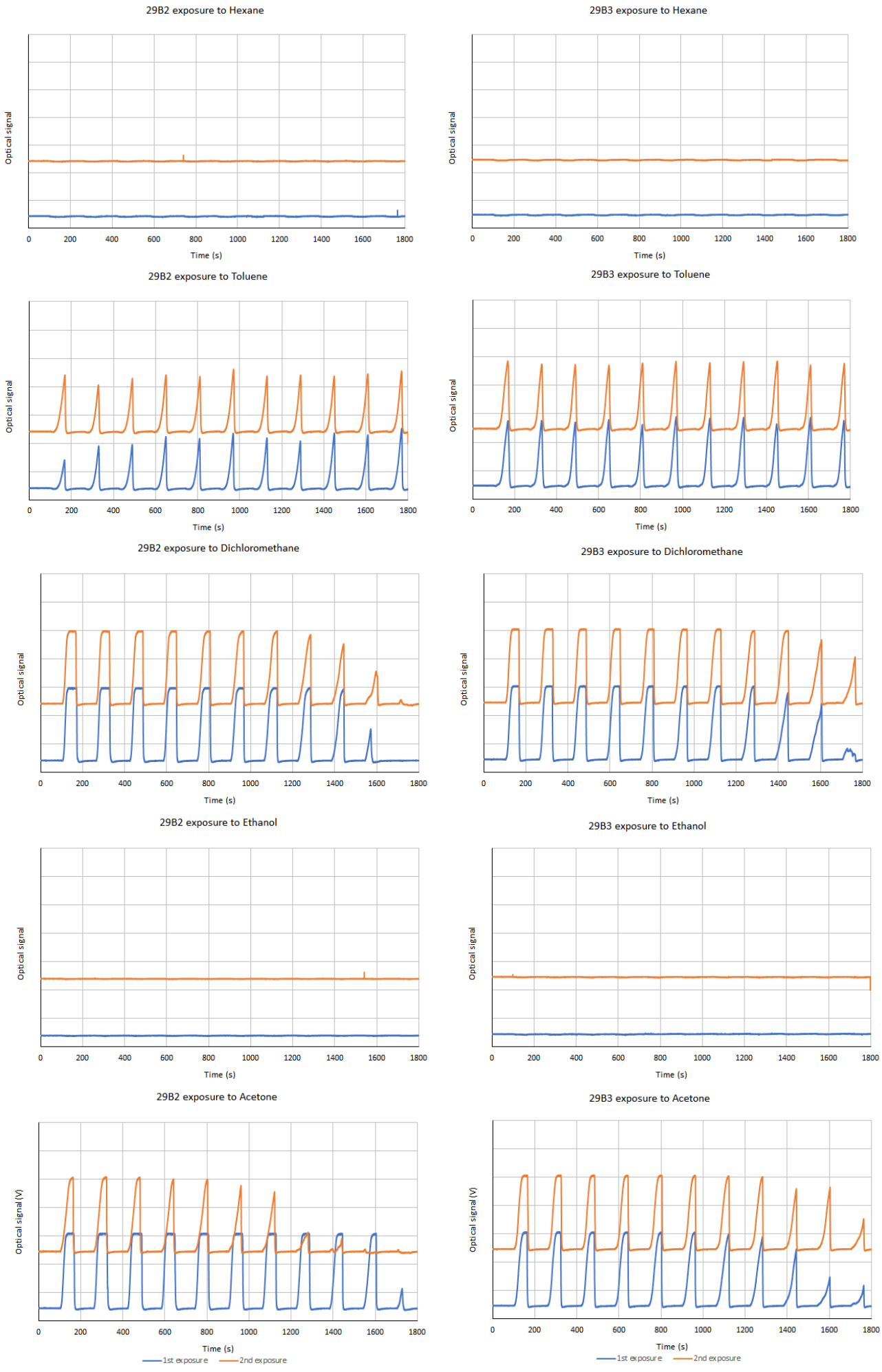

Figure 2.36 - E-Nose tests on films with suberin from 1-hour extraction of cork.

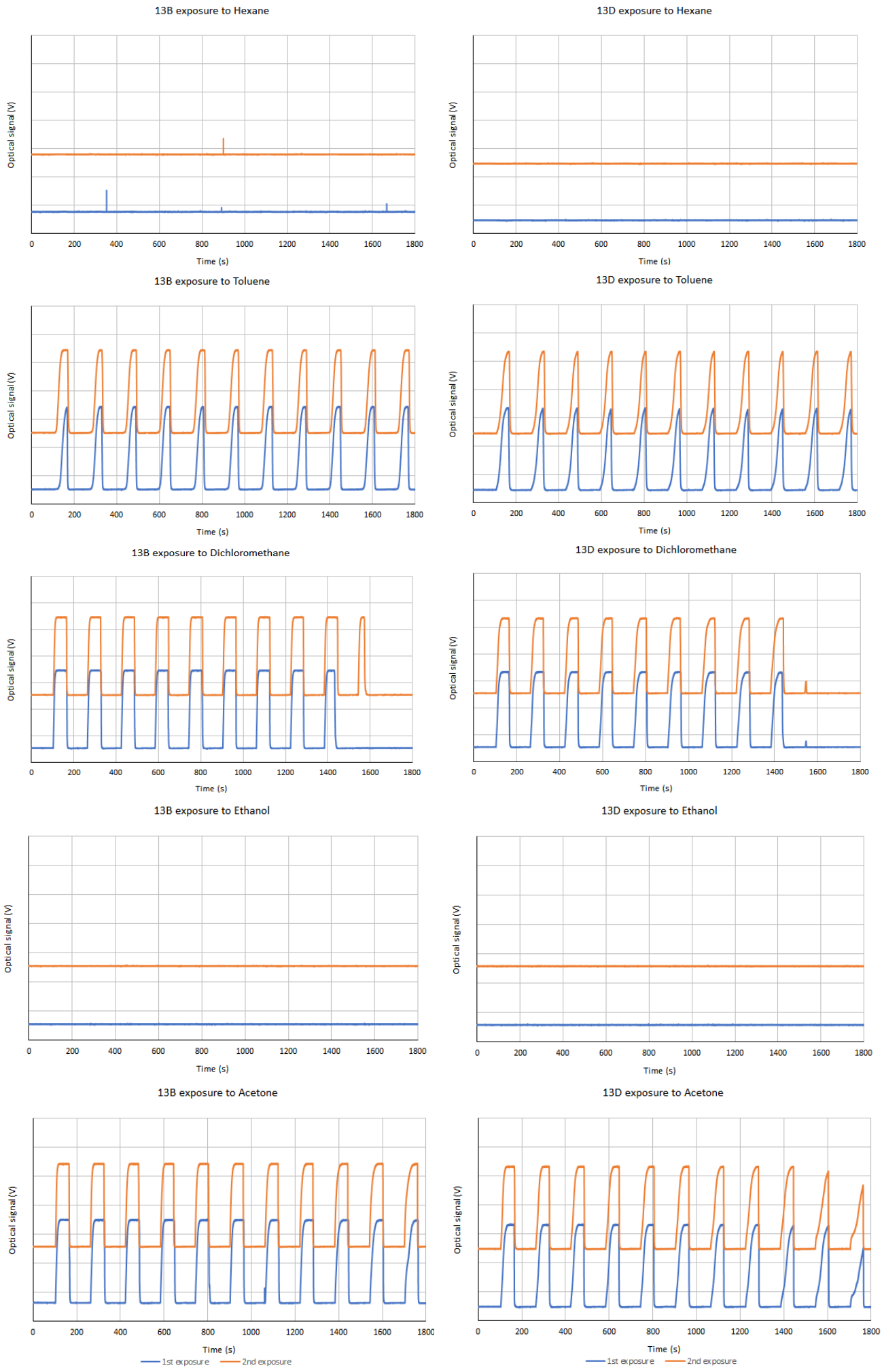

Figure 2.37 - E-Nose tests on films with suberin from 2-hour extraction of cork.

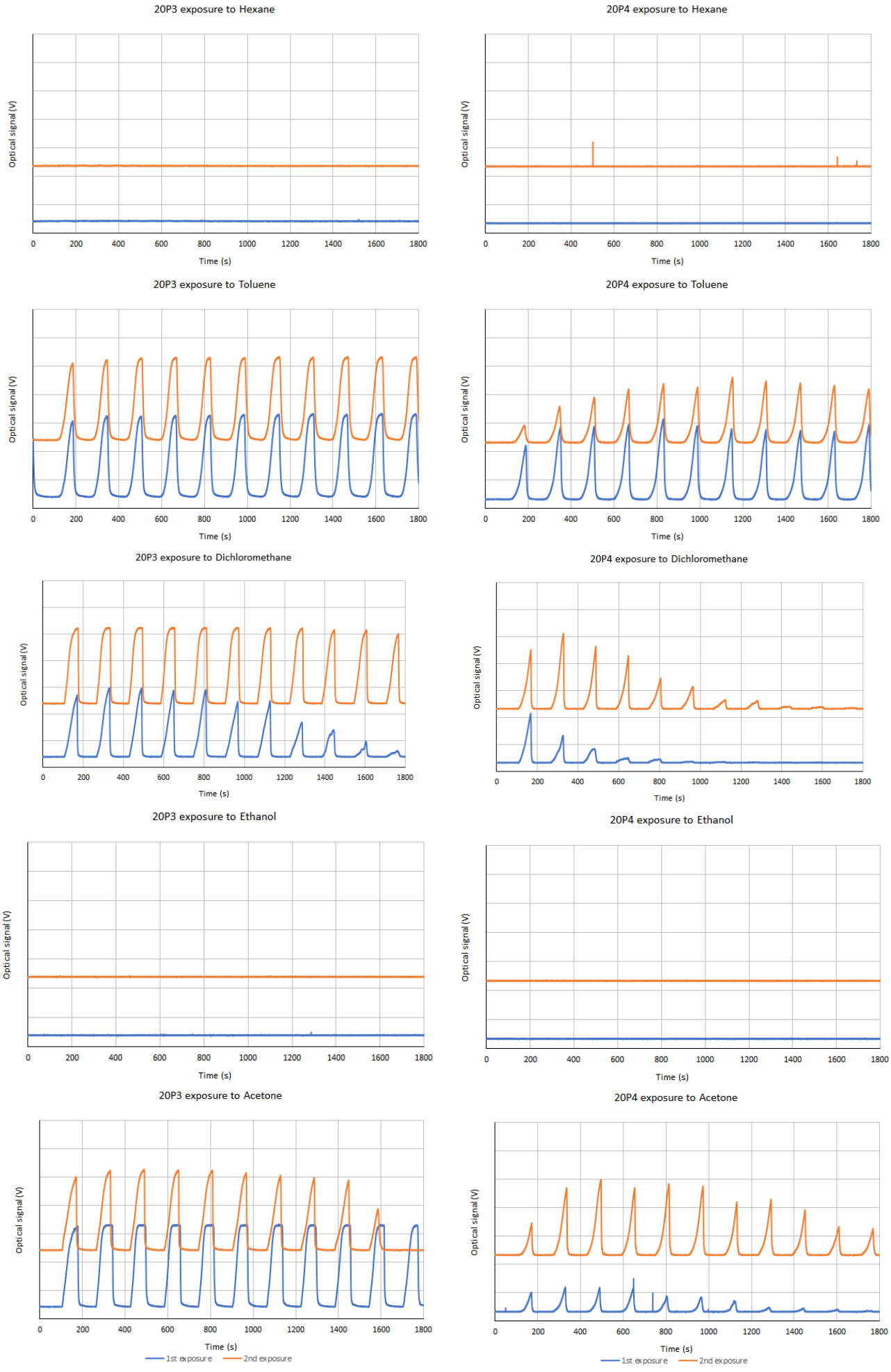

Figure 2.38 - E-Nose tests on films with suberin from 2-hour extraction of white potato skin.

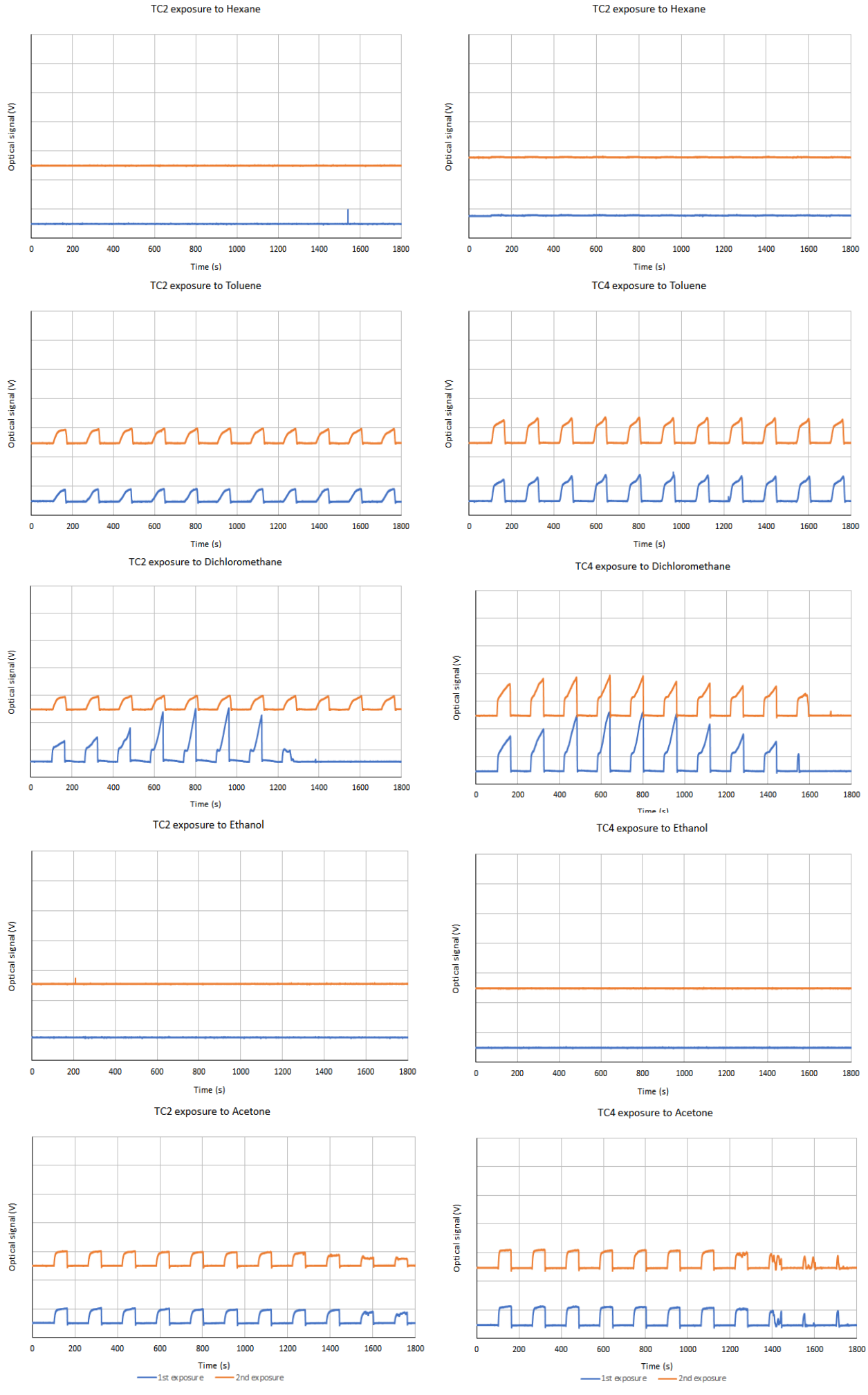

Figure 2.39 - E-Nose tests on films with suberin from 2-hour extraction of tomato skin.

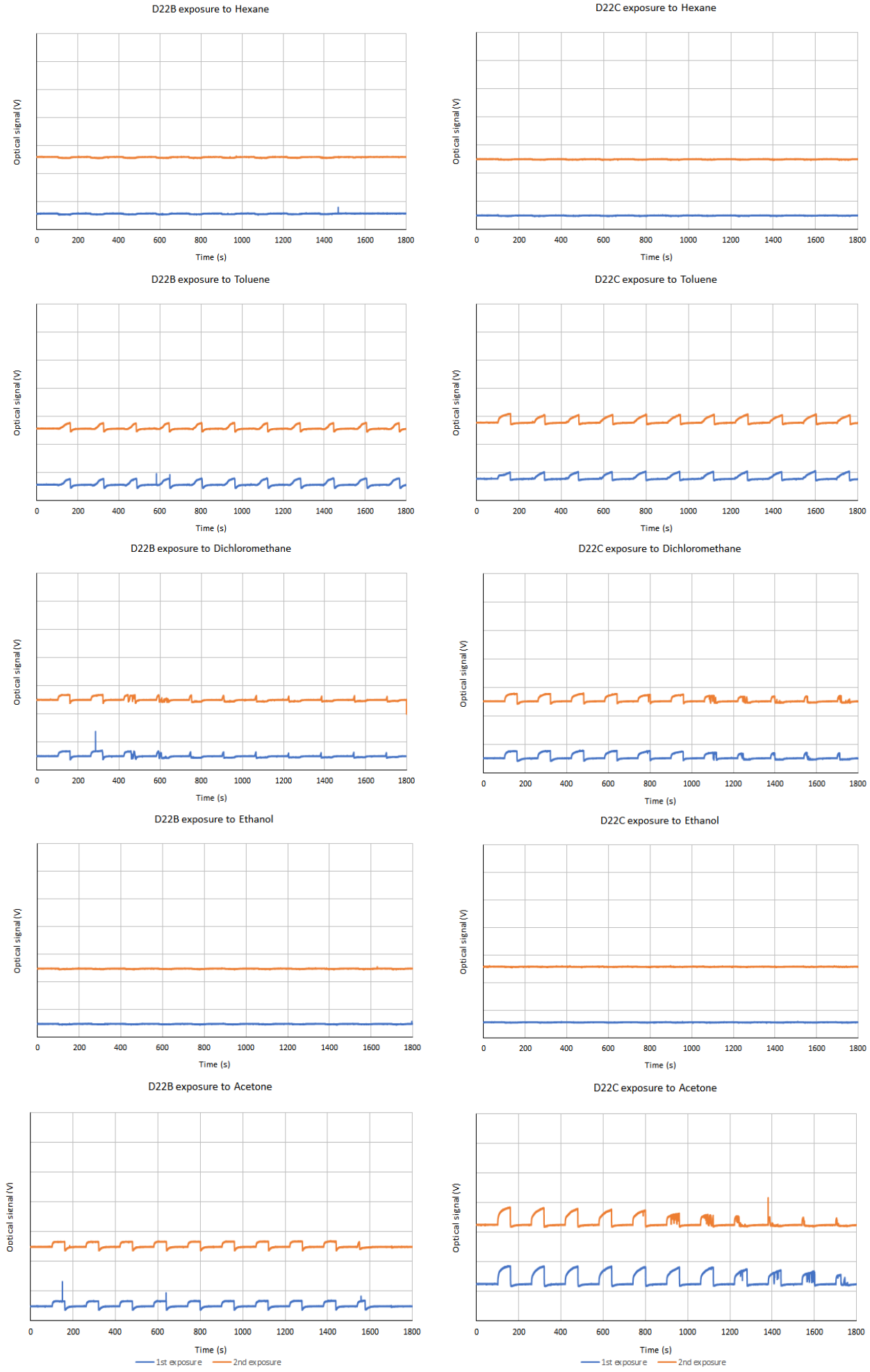

Figure 2.40 – E-Nose tests on films with 1-docosanol.

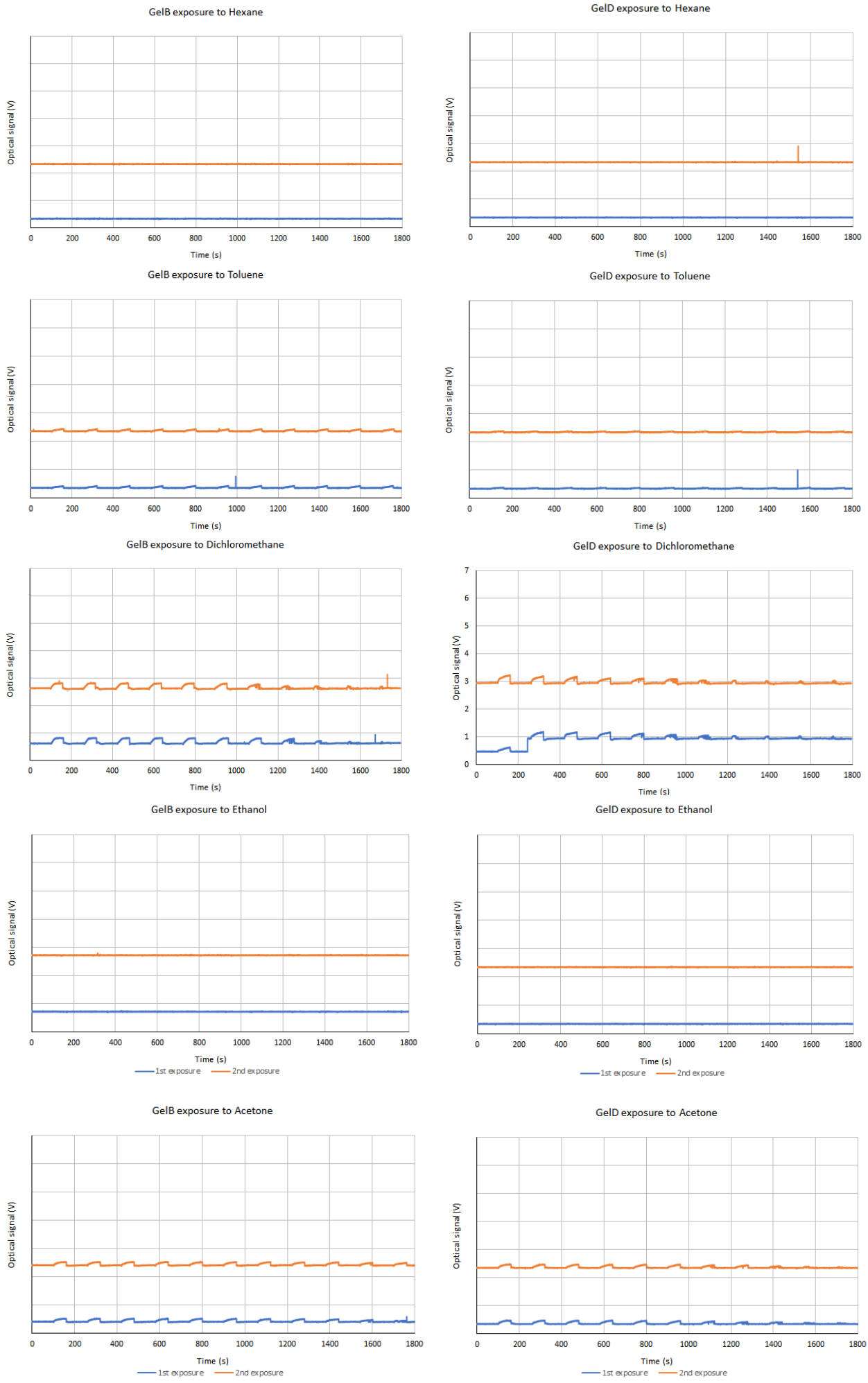

Figure 2.41 - E-Nose tests on films with gelatin from bovine skin.

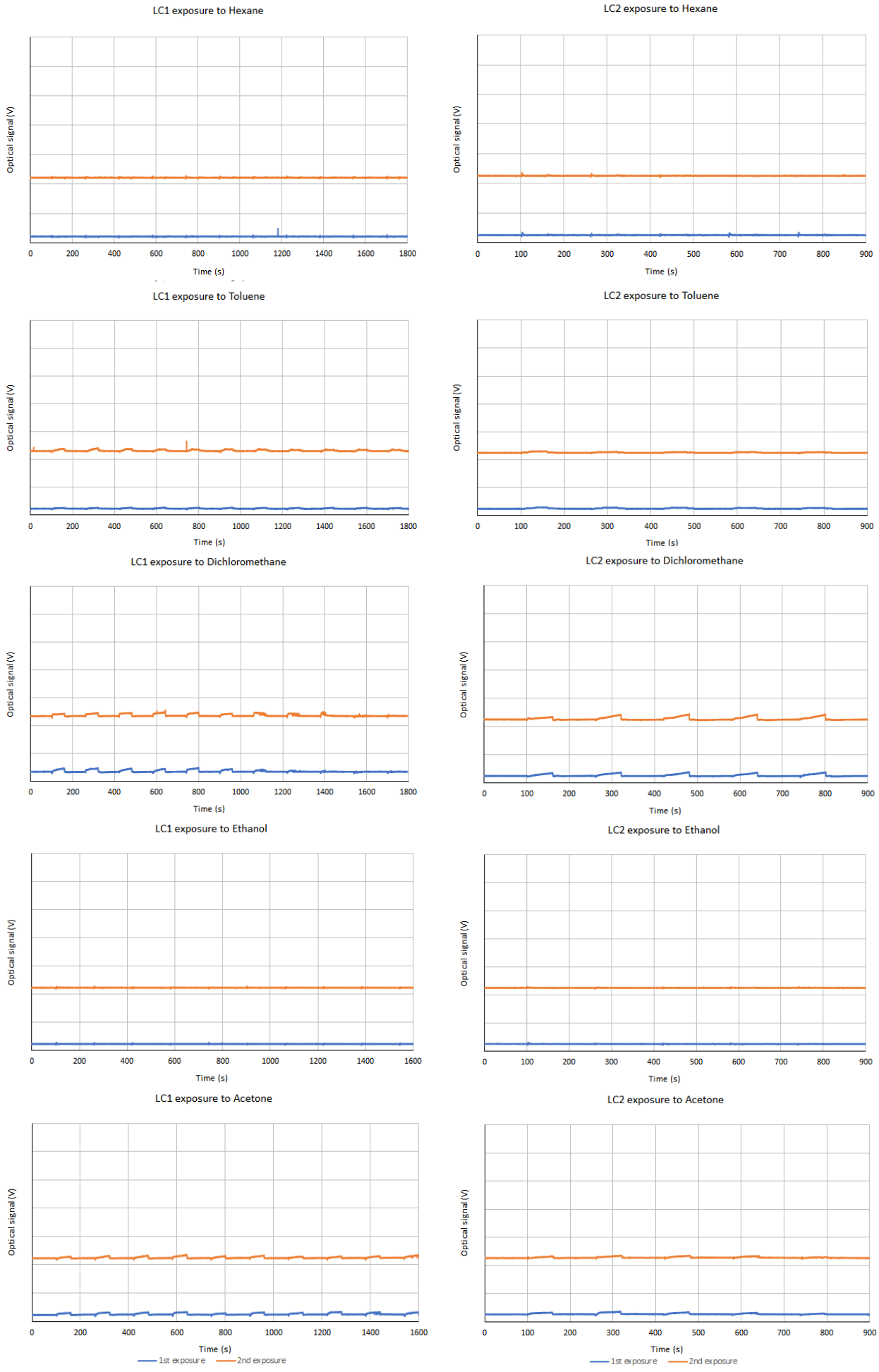

Figure 2.42 - E-Nose tests on 5CB droplets.

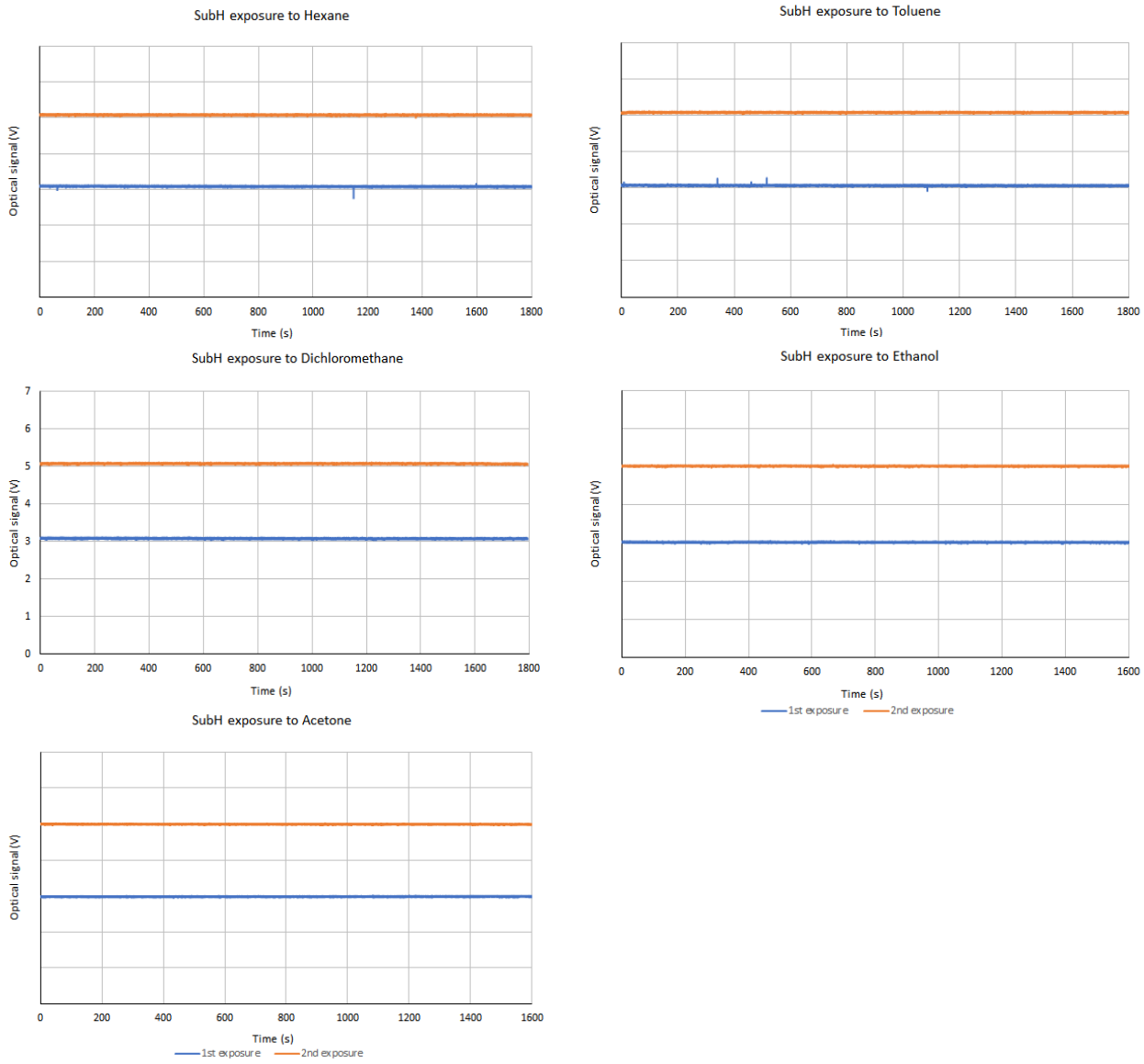

Figure 2.43 - E-Nose tests on films only with suberin from 2-hour extraction of cork (no 5CB).

Gelatin gels (5CB+[BMIM] [DCA]+gelatin)

1. Fill a standard glass vial with milliQ water (tube 1) and heat it to 37ºC, under magnetic stirring (~300 rpm). Use the temperature probe of the hotplate to control the temperature.

   NOTE: The temperature should be kept between 37ºC and 40ºC during all protocol.
2. In the meanwhile, prepare the glass vial without screw neck where the gel will be prepared (tube "ALL"). Choose a magnetic stirrer that occupies the entire diameter of the vial

3. With the tube "ALL" under magnetic agitation (300 rpm):
   - **3.1.** Add 150 µl of [BMIM][DCA] and let it heat for 10 minutes.
   - **3.2.** Add 10 µl of 5CB and leave it mixing for 10 min.
   - **3.3.** Add 50 mg of gelatin and leave it dissolving for 10 – 15 minutes.
     IMPORTANT: the gelatin should be added slowly to avoid the formation of aggregates (approx. 15 mg step by step); it may be necessary to increase the stirring speed up to 500 rpm to facilitate the dissolution of the gelatin.
   - **3.4.** Add 50 µl of water (pre-heated to 37ºC in tube 1) to the mixture and leave it mixing for 5 minutes (300 – 500 rpm)

Figure 2.44 – Protocol for the production of gelatin films.

Table 1.9 – GC-MS calculated contents in suberin extracted from cork and white potato skin.

Families of compounds	Suberin 1-hour cork		Suberin 2-hour cork		Suberin white potato skin	
	%	Sum	%	Sum	%	Sum
Fatty acids	1,18	21,70	0,97	26,08	3,40	13,02
Fatty alcohols	1,39		2,34		0,64	
Hydroxy fatty acids (mono)	8,97		10,66		3,85	
Di- and tri-hydroxy fatty acids	0,66		0,80		0,29	
Epoxy fatty acids	1,50		3,69		0,24	
Alkanedioic acids	7,40		6,74		4,60	
Sterols and triterpenes	0,55		0,83		0,00	
Aromatics and others	0,06		0,05		0,00	
Others (unidentified)	78,30		73,92		86,98	