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# INNOVATIVE MATERIALS AND TECHNIQUES FOR FLAVOR ENCAPSULATION

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# Summary

In this PhD thesis the topic of innovative technologies and materials for the industrial production of encapsulated flavors was addressed.

A commercially available porous starch was evaluated for use as a carrier for liquid flavors in terms of interaction with solvents of different polarity, performance in a finished food product application and protection from oxidation offered to High Oleic Sunflower Oil, using Differential Scanning Calorimetry (DSC), Nuclear Magnetic Resonance (NMR), chemical analyses (SPME/GC-FID, Peroxide Value and Conjugated Dienes value) and sensory analysis. It was found that porous starch has a stronger physical interaction with polar solvents; that flavor retention by porous starch increases with increasing polar affinity between flavor molecule and solvent; that flavor retention in porous starch, in presence of the correct solvent, is equal or higher than flavor retention in a spray dried flavor; that levels of oxidation reached by sunflower oil carried on porous starch is equal or lower to those reached by spray dried oil. The use of porous starch can be an alternative to spray drying for the conversion of liquid flavors to powders.

Different wall materials for spray drying (pea and potato maltodextrins, glucose syrup, gum Arabic, modified starches and yeast β-glucans) and their combinations were studied in terms of retention of diacetyl over time, using a unified method of analysis for direct comparison of data even if produced in different times. Yeast β-glucans were inadequate wall materials for spray drying; pea maltodextrins performed better than potato maltodextrins, but showed a high variability between batches of the same product; glucose syrup caused lower diacetyl retention in all products where it was used in substitution to potato maltodextrin; a commercial modified starch had the highest retention of diacetyl.

Finally, preliminary studies were made for the industrialization of the conjugation reaction between proteins and carbohydrates to produce emulsifiers for flavor emulsion stabilization, exploring: the effect of buffers and ionic strength on the reaction, through Size Exclusion Chromatography

(HP-SEC) and Gel Electrophoresis (SDS-PAGE); the production, through needleless electrospinning, of nanofibers containing proteins and carbohydrates as substrate for the dry state conjugation reaction. These activities are the basis for future work.

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# Introduction

*"...smell and taste are in fact but a single composite sense, whose laboratory is the mouth and its chimney the nose..."*

(Anthelme Brillat-Savarin)

## Flavors

Flavors are those substances and their mixtures which are added to food products with the aim of modifying the original taste and/or smell. Historically, the first flavors to be used were herbs and spices, later on botanical oils and extracts, and finally single molecules, natural or made by synthesis. Nowadays the flavor industry can count on thousands of molecules for the composition of flavors for any food product, be it savory, snack, bakery, confectionery or a beverage[1].

Flavors may be added to industrial foods for different reasons: reintegrating flavor lost during production processes, especially those where heat is involved; standardizing the taste of an industrialized product for consumer satisfaction and to minimize taste variability due to raw material variations; differentiating a product's taste from competitor's analogues; providing products with a flavor that they would be completely lacking otherwise (for example chewing gum and flavored waters).

## Microencapsulation of flavors

Encapsulation is defined as the coating of an active ingredient/material or mixture of materials (core) with an outer layer of different materials (shell or wall)[2].

Encapsulation of active ingredients has been in use for over 50 years in the pharmaceutical, chemical, fragrance and flavor industries and it produces various advantages: a liquid product can be converted to powder form and be thus easier to handle, the core material is isolated from its environment to protect it from evaporation, oxidation and other reactions that can cause its degradation and/or production of off notes, a concentrated product is diluted for ease of use and last but not least, a controlled release of the core material can be obtained[3].

The wall materials used for encapsulation vary depending on the encapsulation technique used, but are generally polymers falling into the classes of starches (including modified starches and dextrins), other carbohydrate polymers such as gum arabic and alginates, and proteins such as whey protein isolates, caseins and gelatin. Lipids are also used as wall materials, for certain applications.

Independently of the encapsulation technique chosen, there are some fundamental characteristics that good wall materials should have, namely they should be inert towards the active ingredient, and protect the core from heat, oxygen and light once in powder form[4].

New wall materials, especially new modified starches and proteins, are constantly being studied with the aim of achieving higher oil loads and above all better controlled release of the encapsulated core material. A wall material that deserves mention is protein-carbohydrate conjugates, obtained through the first steps of Maillard reaction. These products are believed to have excellent emulsifying abilities, which is an important factor in flavor emulsion stabilization prior to encapsulation[5-7]. Before proceeding to their use for encapsulation, however, it is important to evaluate an efficient method for their large scale production[8,9], a topic which is addressed in Part III of this thesis.

**Spray Drying**

Spray drying is the most widespread technique for flavor encapsulation, due to its low costs and available equipment[10]. The process of spray drying was actually developed for the conversion of liquids into powders, for example spray drying of concentrated milk to obtain soluble milk powder. However, it was found that the spray drying of a liquid flavor emulsion produced powder particles that encapsulated the flavor molecules.

Spray drying involves the atomization of a liquid slurry, composed of wall materials, water and the active ingredient, into a drying chamber where it meets hot air which causes the evaporation of water and a dry powder is collected. There are many critical parameters that govern the efficiency and effectiveness of this process.

To begin with, the humidity, flow rate and inlet temperature of the incoming air are important parameters, as they determine the amount of water that can be evaporated from the liquid slurry drops per unit of time and also influence the viscosity of the incoming slurry.

The outlet temperature is also important because it determines the heat stress of the powder, more than the inlet temperature, even though the latter is almost 100°C higher. This is because the evaporation of water during the spray drying process maintains the particles at wet bulb

temperature, whereas when the powder is about to exit the chamber it has a residual humidity of less than 5% and is subjected to the dry bulb temperature. The process temperatures (in and outlet) will also affect the physical form of the finished product[11-13].

The heat stress of the powder is also influenced by the residence time of the product in the drying chamber, which, in turn, is essentially defined by the size of the liquid droplets produced by the atomizer head. Smaller droplets will have a higher surface to volume ratio resulting in faster drying but longer residence time, and larger droplets will have a shorter residence time but slower drying, thus a compromise between all parameters needs to be found.

Last but not least, the composition of the flavor slurry (solids content and viscosity) is important because it influences the amount of water that needs to be dried, the droplet dimension and flavor retention[14,15].

A large body of publications exist that studies the process parameters for spray drying, such as the effect of air properties[16,17], in and outlet temperatures[13,18], slurry composition and atomizer type[11], but it is impossible to define a single optimum operational setup of the spray dryer. Depending on the flavor and wall materials used, and the desired properties of the final product, each recipe will have its optimum parameters that can be decided based on the thorough knowledge of all process variables.

The spray drying technique has been thoroughly studied over the decades, but more research is needed for the selection of new wall materials for the process. Different wall materials are in use for spray drying, the most widespread being gum arabic, maltodextrins, modified starches and milk proteins such as Whey Protein Isolates and casein[19]. The properties which define a good wall material for spray drying are their emulsifying properties for the production of a small sized and stable slurry, their viscosity in solution for slurry pumpability, the ability to retain the active ingredient during atomization and at the same time allow the evaporation of water[4].

The selection of new wall materials aims at finding polymers that are easily available and possibly cheaper than those currently used, while offering the flavor protection from oxidation, heat, evaporation and undesired reactions with other food components[20]. Part of this PhD thesis

focused on exploring the flavor retention of various new wall materials compared to traditional ones (see Part II).

**Porous starch carriers**

A recent application of starch products in the flavor industry is the use of porous starch as a carrier for flavors[21]. This implies a non-classical encapsulation of liquid flavors because one obtains a free flowing powder, however the particles don't have a core-wall structure. The liquid flavor molecules are absorbed into the porous matrix of the starch particles, which act as a sponge. Due to absorption onto porous starch, the vapor pressure of the flavor molecules is reduced, meaning the flavor is maintained within the starch and is slowly released, in equilibrium with headspace flavor concentration.

The use of porous starch to carry flavors requires only a plating procedure, meaning the time and energy consumption necessary for spray drying is saved, resulting finally in a lower cost in use of the powdered flavor[22].

Considering the potential advantages of using porous starch for flavor encapsulation, it was believed worthwhile to dedicate part of this PhD research project to study better its encapsulation efficiency and physical behavior in presence of flavors, the protection offered to the hosted liquid in terms of heat stability and oxidation, and the shelf life of a hosted flavor (see Part I).

**Other techniques for flavor encapsulation**

Besides the search for new wall materials for spray drying, the industry has, over the years, also worked on the development of different techniques for encapsulation, briefly mentioned below[2,20].

Coacervation – this technique involves two oppositely charged polymers in a near stoichiometric ratio that at a correct pH and temperature associate ionically to form microcapsules. The wall is often hardened by chemical or enzymatic crosslinking. The production process is long and costly, and the few existing commercialized products are in a liquid suspension form.

Liposomes – these particles simulate the structure of cells by encapsulating a hydrophilic phase into a lipid double-layer, forming a lypophilic product.

Encapsulation in yeasts – yeast cell walls (β-glucans) may be used in the intact form for the adsorption of flavors or in the hydrolyzed form as spray drying wall materials.

Fluid bed agglomeration – this technique is used to achieve larger and instantly soluble powder particles by wetting fine powders in a fluid bed system and allowing their agglomeration.

Molecular inclusion – this occurs when a small molecule is "hosted" within the lattice structure of a larger molecule, such as β-cyclodextrins.

Spray chilling – this technique is analogous to spray drying but uses low temperatures and fats or oils as wall materials. Products are lypophilic and will release the flavor upon heating and melting.

It must be noted, however, that with few exceptions made for niche products, spray dried powders remain the bulk of commercialized encapsulated flavors.

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# Objective

The objective of this work was the development of new technologies, the improvement of existing technologies and the implementation of new wall materials for the encapsulation of flavors in a specific industrial context.

The research activities were carried out at Kerry Ingredients and Flavors, Parma University and Hohenheim University's laboratories, combining chemical, physical, sensorial and statistical methods of analysis to improve the industry's products.

The first part of this PhD project was the study of a porous starch based carrier to evaluate its applicability for the encapsulation of liquid flavor systems. The second part of this PhD project was the comparison of new and existing wall materials for the encapsulation of flavors by spray drying, in terms of flavor retention, in order to evaluate the implementation of new wall materials. The third part of this PhD project was the production of protein-carbohydrate conjugates for the stabilization of liquid flavor emulsions.

# Part I – Porous Starch for Flavor Encapsulation

## I-A. Evaluation of porous starch as a flavor carrier

This work was presented at the 4th Delivery of Functionality in Complex Food Systems conference in Guelph, Canada, 21-24 August 2011 and is published in Food and Function, 2012, 3 (3), 255 – 261 (C. Belingheri, E. Curti, A. Ferrillo and E. Vittadini).


### Abstract

A commercial porous starch was evaluated for the use as a carrier for liquid flavors. Encapsulation trials performed with diacetyl showed a high initial load and good retention over time when more polar solvents commonly used in flavor creation were used. The physical interactions between the porous starch and solvents used in flavor creation were also studied. The glass transition temperature of the starch decreased upon addition of the polar solvents, ethanol and propylene glycol. Propylene glycol also produced an exothermic peak when mixed with porous starch, possibly due to the formation of complexes between the two components. Low resolution $^{1}$H-NMR results suggested that a stronger interaction was established between more polar solvents and the porous starch, as indicated by a more marked decrease in relaxation times and proton diffusion coefficient of the solvents on adding porous starch.


### Introduction

The encapsulation of flavor molecules is an important operation in the flavor industry, used to prolong flavor shelf-life, with special attention to protecting flavors from undergoing undesired reactions (such as oxidation) and to prevent flavor loss during heat treatments. Since the 1950s the most common technique used to achieve flavor encapsulation in industry is spray-drying, due to the widespread availability of equipment and relatively low cost of operation.[1–3] The spray-drying technique uses various wall materials of polymeric nature, such as gum arabic, maltodextrins and octenyl-succinylated starches as encapsulants.[4,5] The flavor industry is, however, always searching for alternative methods of flavor encapsulation to constantly deliver new products targeted to clients' needs, with new functionalities, and in order to differentiate themselves from competitors.

There are also technical reasons to search for alternatives to spray-dried products, for example the fact that spray-dried flavors are water soluble, limiting their use in fat matrices, and their fast dissolution in the food product on contact with water. The result of this is a short duration of the flavor in the final product, whereas often a sustained release of the flavor is desired.[6]

Alternative techniques to spray-drying, already in use or currently studied by the industry, have been well reviewed.[7,8] The high cost of some of these processes, the difficulty of industrializing them, and the technical difficulties in obtaining stable final products, however, still pose limits to their widespread use.[9,10]

Porous starches have the potential to be used as encapsulation matrices for flavors by applying a simple plating procedure.[11] Plating onto bulking agents, such as maltodextrins or salt, is already in use for the conversion of liquid flavors to powder, however, this does not produce an encapsulated flavor.[12] The use of porous starches for flavor encapsulation would have various advantages. To begin with, the manufacturing cost associated with a plating procedure is less than that associated with a spray-drying procedure, resulting in reduced costs of the encapsulated active material.[13] Moreover, a flavor adsorbed onto a porous matrix could potentially provide a sustained release of the flavor, meaning the headspace of the food product would be constantly refilled with the desired aromatics on successive openings of the product.[11] Furthermore, it could be possible to plate flavors dissolved in solvents that cannot be used in the spray-drying process.

Though some studies have already been performed on the adsorbing capacity of porous starches[14,15] and on the encapsulating ability of porous starches,[16] the nature of the interactions that occur between porous starch and various molecules has not yet been investigated. Furthermore, to the best knowledge of the authors, studies of the performance of a porous starch as a flavor encapsulant have not been reported in the literature.

In this study, the potential use of porous starch matrices for flavor encapsulation by a simple plating procedure is explored. A model molecule (diacetyl) was selected, loaded onto the porous starch and its content in the final product (both fresh and stored) was measured. Furthermore, the

nature of the interaction between the porous starch matrix and the four main solvents used in the flavor industry, which are of different polarity, was studied by analyzing the physical changes that occur upon mixing of the components. This interaction is important considering the high percentage of solvent generally present in a liquid flavor. The solvents studied were, in order of decreasing polarity: ethanol, propylene glycol, triacetin and medium chain triglycerides (MCT).

**Materials and Methods**

*Encapsulated flavor production*

Loading of porous starch - Diacetyl (99.0%, Moellhausen SPA) was dissolved in each of the four selected solvents (ethanol, 96.0%, [Sacchetto SPA], propylene glycol, 99.8%, [Univar SPA], triacetin, 99.0%, [Chemical SPA] and Medium Chain Triglycerides, 99.7%, [MCT; Nutrivis Srl]) and loaded onto the porous starch (StarrierR®, Cargill), using an 80L horizontal body powder mixer equipped with a screw blender (producer unknown). The starch to solvent ratio was 1:1 and the final theoretical content of diacetyl was 0.5%.

Spray Drying - For reference, a spray dried product containing diacetyl was also produced. Diacetyl was dissolved in MCT and spray dried using Gum Arabic (Kerry Ingredients UK Ltd) and maltodextrin (DE 20 potato maltodextrin; Brenntag SPA) as wall materials, at 40% solids, using a single stage spray dryer (APV, Italy; Tin = 160°C; Tout = 90°C). The theoretical diacetyl content of the finished product was 0.5%.

*Diacetyl content*

A Solid Phase Micro Extraction (SPME) method was developed to quantify the diacetyl present in each product. 0.5g of sample was weighed into a vial for SPME together with 2g of salt, 10g of deionized water and 20-50µL of Internal Standard solution (ethyl butyrate, 99.9%, [Frutarom]). The vial was equilibrated for 10 minutes at 30°C in a 400ml water bath under magnetic rotation at 1500rpm, and then a syringe for SPME (100µm PDMS fiber, Supelco) was exposed to the headspace for 10 minutes at the same conditions. The fiber was then injected into a Gas Chromatograph equipped with DB1 and DB1701 columns and a Flame Ionization Detector (GC 6890,

Agilent; Injector T = 280°C; splitless mode; T1 = 40°C for 3 minutes; ramp 10°C/min to 280°C; final T = 280°C for 5min; detector T = 300°C). Each sample was analyzed at least in triplicate.

*Starch – solvent interactions*

To study the physical interactions occurring between starch and ethanol, propylene glycol, triacetin and MCT, starch/solvent mixtures of varying ratios were studied: a) 0.0% solvent; b) 16.7% solvent (83.3% starch); c) 33.3% solvent (66.7% starch); d) 60.0% solvent (40.0% starch); e) 100.0% solvent. Samples in graphs and tables are identified based on the solvent content.

Thermal properties - Differential Scanning Calorimetry (DSC) - 8 to 20 mg of sample were weighed into a stainless steel sample pan (Perkin Elmer, Somerset, NJ, USA) and compressed using a flat bottomed metal rod to maximize heat transfer through the material. The pan was hermetically sealed and placed in the DSC furnace. An empty sealed pan was used as reference. The Differential Scanning Calorimeter (DSC Q100, TA Instruments, Newcastle, DE, USA) was calibrated with indium and mercury. Samples were cooled to -15°C and then heated to 200°C at 15°C/min. At least triplicate analysis of each product was carried out.

DSC thermograms were analyzed using a Universal Analysis Software, Version 3.9A (TA Instruments, New Castle, DE). The following parameters were obtained: glass transition temperature and glass transition onset and offset temperatures where Tg was present; peak temperature, peak enthalpy and peak onset and offset temperatures, where a peak was present.

$^{1}$H-NMR - A bench-top low resolution (20 MHz) $^{1}$H NMR spectrometer (the MiniSpec, Bruker Biospin, Milano, Italy) operating at 25°C was used to study proton molecular mobility by measuring the free induction decay (FID), transverse (T2) and longitudinal (T1) relaxation times and self diffusion coefficient (D). Samples were inserted into a 10 mm NMR tube and compacted on the bottom to obtain ~2 cm high samples. Tubes were sealed with Parafilm® to prevent moisture loss during the NMR experiment and placed in the NMR for 5 minutes to equilibrate to 25°C prior to analysis.

FID decay curves were acquired using a single 90° pulse, followed by dead time of 7 μs and a recycle delay of 0.6-10 s depending on the sample. T2 (transverse relaxation times) were obtained with a CPMG pulse sequence[17,18] with a recycle delay of 0.6-10s and 6000-12000 data points depending on the sample. T1 (longitudinal lattice relaxation times) were determined by the inversion recovery pulse sequence with an interpulse spacing ranging from 0.1 to 2500ms, a recycle delay of 0.6-10s depending on the sample and 20 data points. T2 and T1 curves were analyzed as quasi-continuous distributions of relaxation times using UPEN software (UpenWin© version 1.04, Alma Mater Studiorum – Bologna University, Italy).

The proton self diffusion coefficient (D) was obtained, at 25°C, with a pulsed-field gradient spin echo (PFGSE) pulse sequence[19]. The instrument was calibrated with pentanol (self diffusion coefficient = 0.29*10-9 m2/s at 25°C).

*Statistical Analysis*

All data was statistically evaluated by one way analysis of variance (ANOVA) and a post hoc test (LSD, $\alpha<0.05$) using SPSS Statistics software (versions 17.0 and 19.0, IBM Corporation, Armonk, NY, USA). Where applicable, a multifactor analysis of variance was applied.

## Results and discussion

*Loading of flavor onto porous starch*

Diacetyl was successfully loaded onto the porous starch by applying a simple plating procedure and a dry and homogeneous product was obtained within 7 min of mixing. The processing time to obtain the spray dried control was over an hour. The level of diacetyl incorporated into the porous starch, expressed as a percentage of the theoretical total, was: 63.42 ± 4.13% when the solvent was ethanol; 90.41 ± 5.43% with propylene glycol; 78.73 ± 7.10% with triacetin and 64.37 ± 5.24% with MCT (Figure 1). The spray dried control contained 53.56 ± 6.07% of the theoretical total of diacetyl.

A multifactor analysis of variance performed on this data showed that both the type of solvent used, as well as the shelf life time, had a significant

influence on the diacetyl content of the products ($p<0.05$, see data in Table 1). As far as the effect of the solvent is concerned, the product containing propylene glycol had the highest diacetyl content, independent of the time of conservation, followed by the product containing ethanol, the product containing triacetin which was not significantly different from the spray dried product, and finally the product containing MCT. Higher diacetyl contents in the final product were thus measured with increasing polarity of the solvent, with the exception of ethanol, probably due to its high volatility causing losses during processing. Increased flavor retention with increased polarity of the flavor molecule has previously been reported[20], and this also seems to hold based on the polarity of the solvent present.

The effect of time was also significant for the quantification of diacetyl, as shown in Table 1. A significant decrease of diacetyl content is shown over time, independent of the solvent used. Not all products, however, showed the same rate of decrease over time, as is shown in Figure 1. After 6 months of shelf life, the diacetyl content had significantly decreased for all porous starch based products, but more markedly in the presence of triacetin and MCT (Figure 1). The spray dried control only showed minimal losses of diacetyl content over 6 months of storage. Products with ethanol seemed to better retain diacetyl during the first 3 months of storage, and those with propylene glycol did not show a decrease in diacetyl content between 3 and 6 months of storage and, after 6 months, the diacetyl content for these products was still higher than for the spray dried product.

Considering the reduced production times and costs, the higher initial flavor load and the satisfactory flavor retention (especially in presence of polar solvents), the porous starch evaluated here has very interesting potential to be used as a carrier for flavors.

*Starch – solvent interactions*

The DSC thermogram for pure starch (water content ~ 9% on wet basis) showed the presence of a glass transition in the temperature range 49 – 68°C (onset – offset temperatures), with a mid-range value of 59 ± 4°C (Figure 2A).

Both the addition of ethanol and propylene glycol to the starch produced a significant decrease in the mid-range values of Tg, independent of the amount added, with propylene glycol decreasing the Tg significantly more than ethanol. The addition of triacetin and MCT had no significant effect on starch mid-range Tg (Table 2 and Figure 2B). The amount of solvent added was also important in defining a decrease in Tg, but as Figure 2B shows, this was significant only for propylene glycol. Starch/solvent mixtures at 60.0% or 100.0% solvent did not show a Tg in the temperature range considered in this study.

The temperature range for glass transitions (difference between onset and offset temperature) remained between 18 and 22°C for all samples, with the exception of starch/propylene glycol mixtures whose range was narrower (9-12°C). A decrease in starch Tg possibly indicates an increased mobility of the starch chains on interaction with polar solvents, due to a plasticization effect of small molecules such as ethanol and propylene glycol, as has been previously reported[21,22].

Samples containing both starch and propylene glycol also displayed an exothermic peak upon heating (Figure 3). The peak temperature was 74 ± 2°C for 16.7% solvent, 82 ± 3 °C for 33.3% solvent and 103 ± 10 °C for 60.0% solvent, the latter resulting significantly higher than the previous two values ($p<0.05$). Peak onset and offset temperatures followed the same pattern as peak temperatures and were, respectively, 56 ± 4 °C and 106 ± 5 °C for 16.7% solvent, 63 ± 6 °C and 105 ± 2 °C for 33.3% solvent and 78 ± 12 °C and 122 ± 10 °C for 60.0% solvent. The enthalpy content of the peak was not significantly different for all three samples (9 ± 2 J/g, 8 ± 1 J/g and 6 ± 3 J/g for samples containing 16.7%, 33.3% and 60.0% propylene glycol, respectively). This exothermic peak is probably due to the formation of complexes between starch and propylene glycol, a phenomenon previously documented in literature[23,24], and indicative of a strong physical interaction between this solvent and the porous starch.

Proton Free Induction Decays ($^1$H FID) allowed the study of the more rigid portion of the sample. $^1$H FID curves ($t < 0.1$ ms) were comparable among the four solvents, the signal hardly decreased due to the fact that solvent protons are very mobile. On addition of starch, curves of all samples became progressively steeper, due to the presence of the starch molecules

that had a higher rigidity. $^{1}H$ FID decays in samples containing the same percentage of solvent were comparable and not affected by the solvent type. Typical curves for pure solvent and all starch/solvent ratios are shown in Figure 4. The presence of solvents did not seem to influence the relaxation of the rigid protons in the starch chains in the time relaxation window provided by this experiment.

$^{1}H$ $T_2$ mobility of pure solvents was, on the contrary, found to be quite different as shown by the $^{1}H$ $T_2$ distributions of relaxation times (large and small dashed lines in Figures 5A-D). Ethanol (Figure 5A) and propylene glycol (Figure 5B) showed a unimodal distribution of relaxation times characterized by a peak maximum at ~1541ms and ~110ms respectively. Triacetin (Figure 5C) showed a heterogeneous proton distribution with a minor $^{1}H$ population (~3% of protons) relaxing around 100ms and the bulk of solvent (~97%) relaxing at ~250ms (peak maximum). The large peak was not symmetrical in shape but showed a 'tail' at higher relaxation times. MCT (Figure 5D) had two resolved $^{1}H$ populations both represented by a narrow peak with relaxation maxima at ~80ms (~13% of protons) and ~240ms (~87% of protons) respectively as previously reported[25].

For all solvents, a $^{1}H$ $T_2$ peak with relaxation maximum between 0 and 1 ms was observed on the addition of porous starch. This peak increased in percentage as the starch content increased (from less than 6% of the total proton population at the lowest starch content, to ~30% at the highest starch content) and was similar in shape for all solvents, it was therefore tentatively attributed to starch protons.

As far as the solvent peaks are concerned (relaxation time distributions for pure solvents), on addition of porous starch, $^{1}H$ $T_2$ relaxation time maxima for MCT did not substantially change, as shown in Figure 5D, whereas in the aforementioned study[25] the authors found a strong decrease in $^{1}H$ $T_2$ relaxation times after adsorption of MCT onto a porous carrier and attributed this decrease to interactions occurring between the solvent and the carrier. It must be taken into account that no details about the experiments are given in the cited study[25] and, therefore, the conflicting results could be due to different experimental conditions. It seems in our case, however, that the $^{1}H$ $T_2$ mobility of MCT is not being influenced by the presence of porous starch. Similarly, the $^{1}H$ $T_2$ distribution

of triacetin was hardly affected by the addition of starch (Figure 5C), suggesting little or no interaction between triacetin and starch, observable in this NMR mobility time frame. In the case of ethanol and propylene glycol, on the contrary, strong and constant decreases in $^1H$ $T_2$ relaxation times occurred on addition of increasing quantities of porous starch (Figures 5A and 5B). The $^1H$ $T_2$ relaxation times (solvent peak maximum) for samples containing ethanol and propylene glycol are shown in Table 3. For propylene glycol, both the shift of the peak maximum to shorter relaxation times, as well as a broadening of the peak were observed. A fairly strong interaction between starch and propylene glycol may be hypothesized as there is a strong reduction of relaxation times indicating a reduced mobility of propylene glycol protons in the presence of starch. In the case of ethanol, not only a shift of peak maximum to shorter relaxation times is observed on the addition of porous starch, but there is also the appearance of a tail to the main peak, towards shorter relaxation times, and the tail dimensions increase with increasing starch content. The presence of the tail might possibly indicate that some solvent protons (slower relaxing population) became less and less mobile upon the addition of starch, but they are still interacting with the bulk solvent in the $T_2$ NMR timeframe.

$^1H$ $T_1$ distributions of relaxation times (Figure 6A) were unimodal and comparable in shape for all solvents. Representative $^1H$ $T_1$ relaxation times were similar for propylene glycol, triacetin and MCT (peak maximum around 200ms). Ethanol showed longer relaxation times (peak maximum at 1750ms) indicating a higher proton mobility. On addition of starch, $^1H$ $T_1$ distributions of relaxation times retained their unimodal shape but tended to broaden towards shorter relaxation times, with the exception of MCT where no changes occurred, and most markedly for ethanol where the largest differences were observed (Figure 6B). The peak base width went from around one order of magnitude for pure ethanol to almost three orders of magnitude for the samples containing 33.3% and 16.7% ethanol. The peak for the sample containing 16.7% ethanol no longer showed a maximum but had a flat top. The broadening of the peak indicates an increased heterogeneity in proton mobility of the sample. The protons have different mobility and relaxation times but are not independent populations as they

somewhat interact in the time frame of this experiment and are therefore not resolved into separate peaks.

Considering the fact that $^{1}H$ $T_2$ and $^{1}H$ $T_1$ relaxation times are a measure of molecular mobility, with increasing times corresponding to increasing proton mobility[26], it seems that the mobility of the two polar solvents (ethanol and propylene glycol) is being reduced in the presence of porous starch, probably due to the interactions occurring between the solvent molecules and the starch chains.

The proton self diffusion coefficient (D) measures the translational mobility of protons in the sample. The D value of samples was shown to be significantly influenced by the type of solvent present, indicating that the different solvents have a different translational mobility (Table 4). The D value of samples was also significantly decreased by subsequent additions of starch to the mixture, indicating that the presence of starch significantly influences the mobility of the solvents (Table 4).

As is shown in Table 5, the D value of pure ethanol was much higher than the D value of the other solvents and significantly decreased on addition of starch. This indicates that the translational mobility of protons in the ethanol/starch mixture is significantly reduced, even when ethanol represents the largest fraction of the sample (60.0%). The D value of the other solvents significantly decreased on addition of porous starch, mainly when starch composed the largest fraction of the sample. These results may indicate that the nature of the interactions between the starch and the solvents is not only sterical (dependant on the starch's microstructure), because the mobility of the apolar solvents was not greatly reduced even though they are larger molecules. Ethanol's translational mobility is reduced probably due to polar interactions with the starch chains. A D value for pure starch was not measurable due to the high rigidity of the sample and the lack of translational mobility of the starch molecules.

## Conclusions

The results obtained in this study show the potential applicability of porous starch as a flavor carrier. The polarity of solvents was a key factor in determining the higher flavor molecule content over time as ethanol and propylene glycol showed the lowest losses during storage. The more polar solvents, ethanol and propylene glycol, were also found to interact more strongly with the porous starch as evidenced by DSC and molecular mobility measurements ($^1$H-NMR). It will be interesting in the future to investigate the performance of the final flavor product into real food systems.

**List of Tables**

Table 1. Diacetyl content (% of theoretical total) of porous starch based products and spray dried control – multifactor ANOVA showing effect of type of solvent and effect of shelf life time. A different letter means a significant difference of diacetyl content ($p<0.05$).

| **Solvent** | **Ethanol** | **Propylene glycol** | **Triacetin** | **MCT** | **Spray dry** |
|---|---|---|---|---|---|
| **Average** | 52.96 [b] | 77.53 [a] | 48.07 [c] | 37.58 [d] | 48.95 [c] |
| **Standard Deviation** | 11.80 | 11.80 | 23.93 | 22.82 | 6.32 |
| | | | | | |
| **Time** | **Fresh** | | **3 months** | | **6 months** |
| **Average** | 65.48 [a] | | 46.56 [b] | | 40.57 [c] |
| **Standard Deviation** | 15.25 | | 14.96 | | 17.40 |

Table 2. Mid-range glass transition temperature (°C) of starch:solvent mixtures - multifactor ANOVA showing effect of type of solvent and effect of amount of solvent. A different letter means a significant difference in glass transition temperature ($p<0.05$).

| **Type of Solvent** | **Ethanol** | **Propylene glycol** | **Triacetin** | **MCT** | **No Solvent** |
|---|---|---|---|---|---|
| **Average** | 38.48 [b] | 26.43 [c] | 58.79 [a] | 55.00 [a] | 58.62 [a] |
| **Standard Deviation** | 5.37 | 9.05 | 3.01 | 0.24 | 4.21 |
| | | | | | |
| **Amount of Solvent** | **0.0%** | | **16.7%** | | **33.3%** |
| **Average** | 58.62 [a] | | 45.88 [b] | | 41.85 [c] |
| **Standard Deviation** | 4.21 | | 11.12 | | 17.78 |

Table 3. $^{1}H$-$T_2$ relaxation times (peak maximum) for starch/ethanol and starch/propylene glycol mixtures (ms).

| | **Ethanol** | **Propylene glycol** |
|---|---|---|
| **Pure solvent** | 1541 | 110 |
| **60.0% solvent** | 827 | 59 |
| **33.3% solvent** | 451 | 39 |
| **16.7% solvent** | 287 | 26 |

Table 4. Proton Self Diffusion Coefficients ($D*10^{-9}$ $m^2/s$) of starch:solvent mixtures - multifactor ANOVA showing effect of type of solvent and effect of amount of solvent. A different letter means a significant difference in glass transition temperature ($p<0.05$).

| **Type of Solvent** | **Ethanol** | **Propylene glycol** | **Triacetin** | **MCT** |
|---|---|---|---|---|
| **Average** | 0.830 $^{a}$ | 0.055 $^{c}$ | 0.081 $^{b}$ | 0.045 $^{c}$ |
| **Standard Deviation** | 0.088 | 0.009 | 0.018 | 0.012 |
| | | | | |
| **Amount of Solvent** | **16.7%** | **33.3%** | **60.0%** | **100.0%** |
| **Average** | 0.206 $^{d}$ | 0.245 $^{c}$ | 0.255 $^{b}$ | 0.281 $^{a}$ |
| **Standard Deviation** | 0.290 | 0.323 | 0.351 | 0.380 |

Table 5. Proton Self Diffusion Coefficients ($D*10^{-9}$ $m^2/s$) of starch/solvent mixtures. A different letter within a row means a significant difference of D at variable amounts of solvent in the starch/solvent mixture ($p<0.05$).

| **% solvent** / **Solvent** | **16.7%** | **33.3%** | **60.0%** | **100.0%** |
|---|---|---|---|---|
| **MCT** | 0.034±0.007$^{b}$ | 0.041±0.005$^{b}$ | 0.044±0.011$^{b}$ | 0.056±0.013$^{a}$ |
| **Triacetin** | 0.055±0.015$^{b}$ | 0.082±0.012$^{a}$ | 0.087±0.015$^{a}$ | 0.094±0.011$^{a}$ |
| **Propylene glycol** | 0.044±0.005$^{b}$ | 0.063±0.007$^{a}$ | 0.059±0.006$^{a}$ | 0.051±0.005$^{b}$ |
| **Ethanol** | 0.691±0.050$^{d}$ | 0.791±0.029$^{c}$ | 0.866±0.018$^{b}$ | 0.925±0.024$^{a}$ |

**List of Figures**

Figure 1. Diacetyl content of porous starch products and a spray dried product, expressed as percentage of the theoretical total, at the time of production (black bars) and after 3 (grey bars) and 6 (white bars) months. A different letter within a solvent group means a significant difference in diacetyl content over time ($p<0.05$).

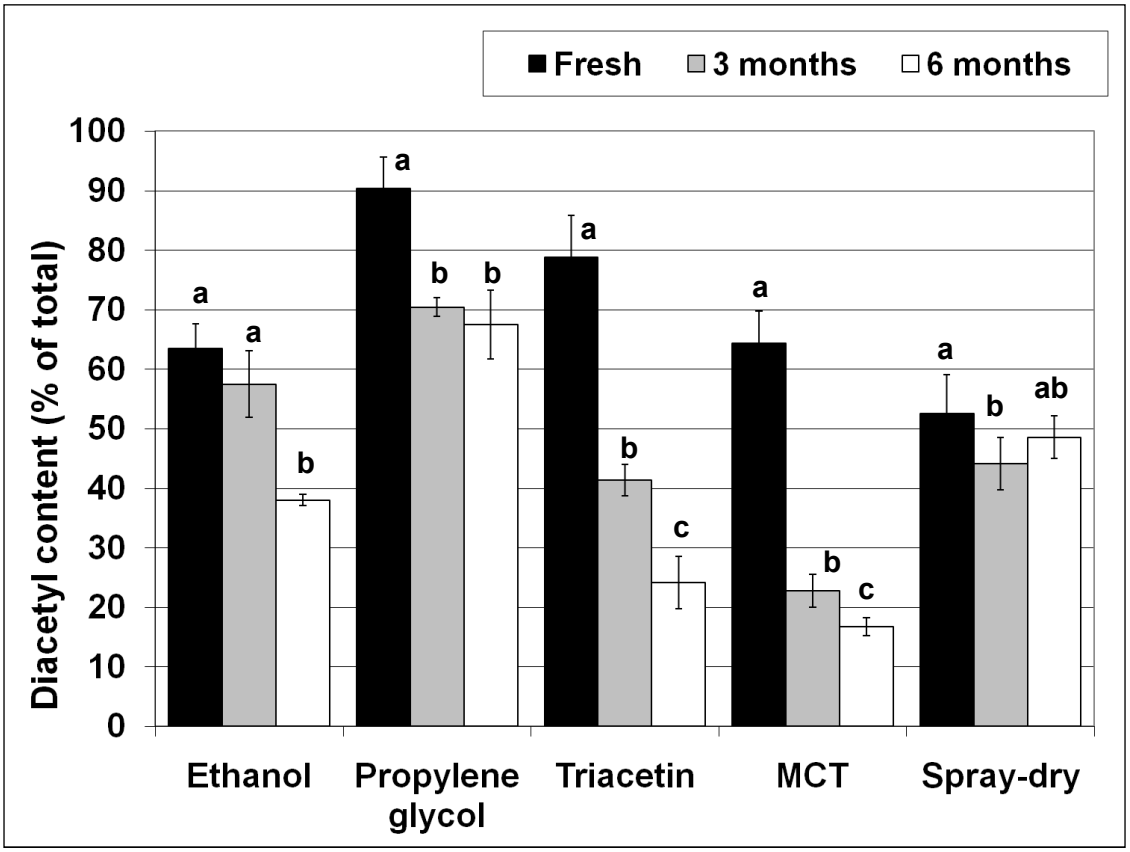


Figure 2A. Characteristic DSC thermogram for porous starch in the 0 – 180°C range showing the glass transition.

Figure 2B. Mid-range glass transition temperatures (Tg) for starch/solvent mixtures. A different letter along a solvent line means a significant difference of Tg for different starch/solvent mixtures ($p<0.05$).

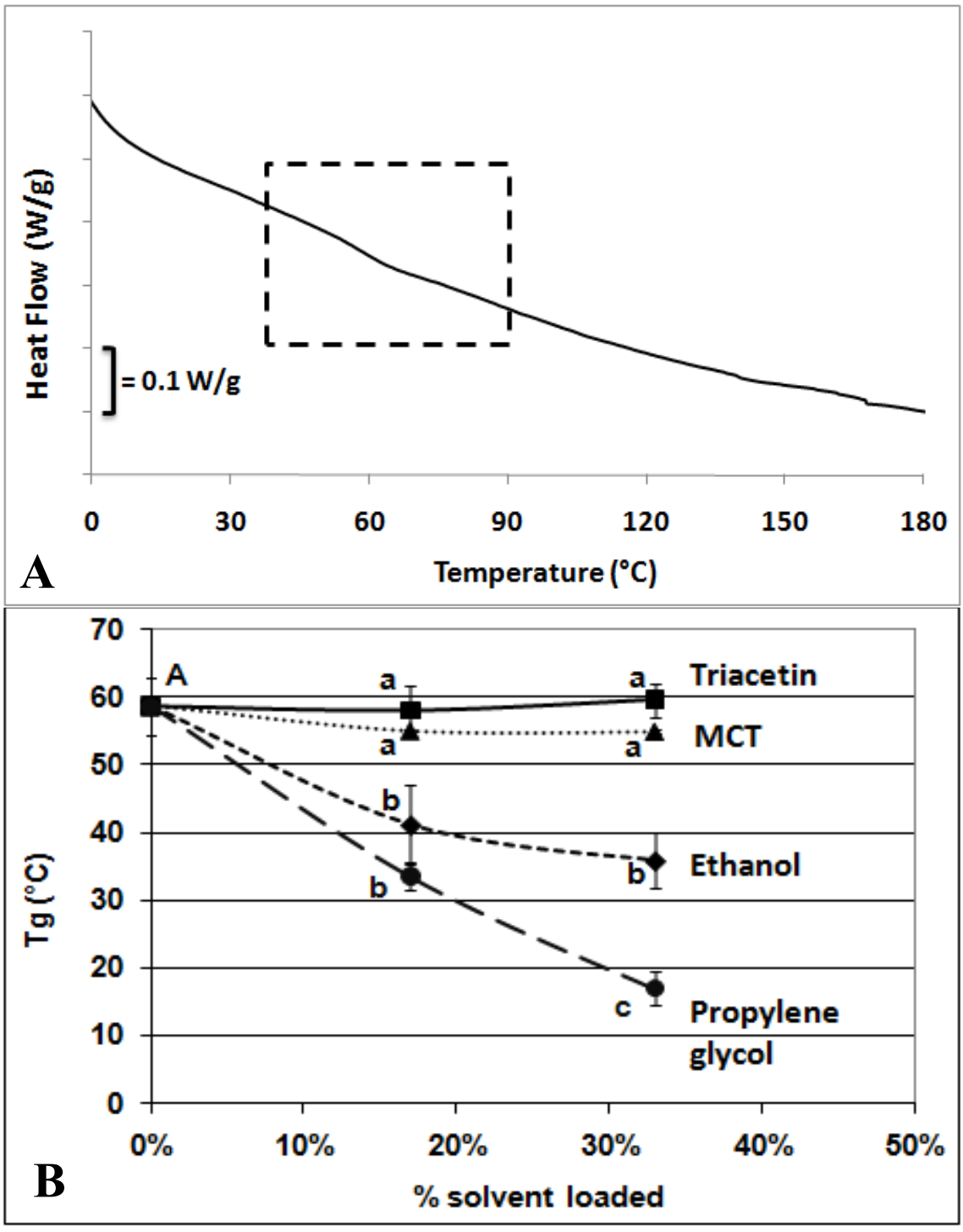


Figure 3. DSC thermograms for starch/propylene glycol mixtures in the 0 – 180°C range.

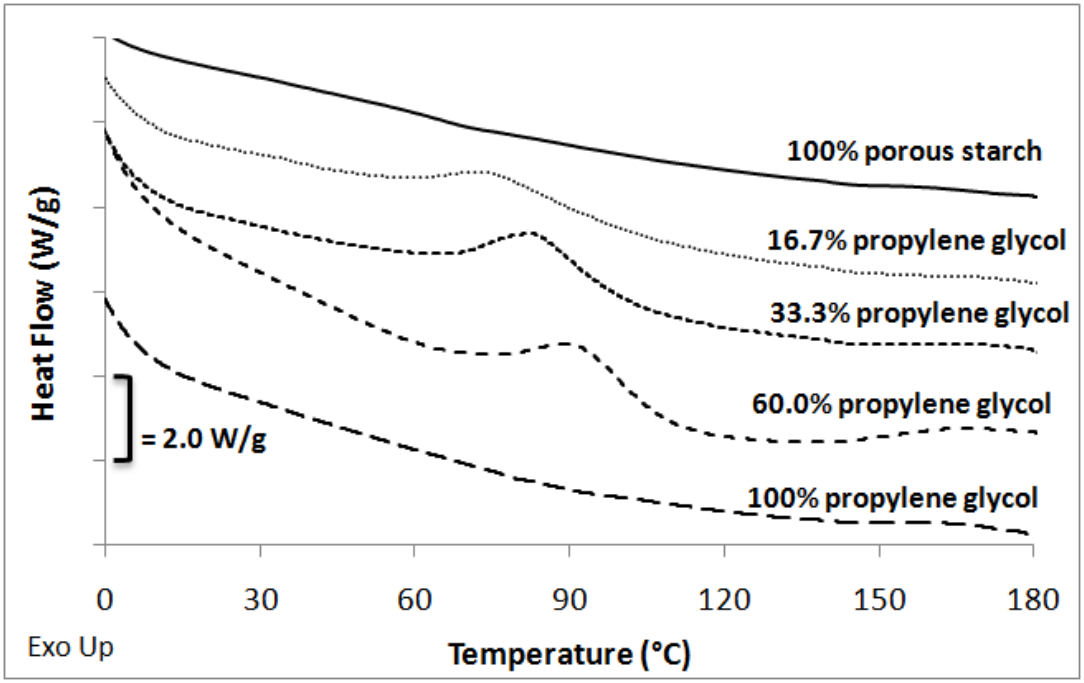



Figure 4. Typical $^{1}H$ FID decays for starch/solvent mixtures, t < 0.1ms (dotted line = 16.7% solvent; large dashed line = 33.3% solvent; large and small dashed lines = 60.0% solvent; solid line = pure solvent).

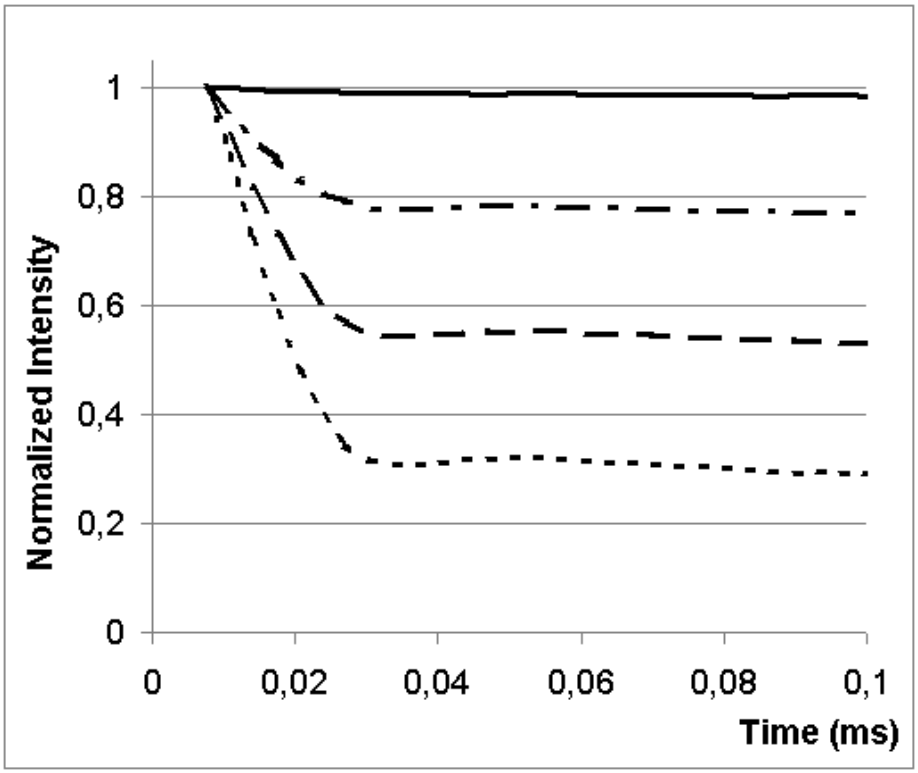


Figure 5. Proton transverse relaxation times (1H T2) for starch/solvent (A = ethanol; B = propylene glycol; C = triacetin; D = MCT) mixtures at different ratios (dotted lines = 16.7% solvent; solid lines = 33.3% solvent; large dashed lines = 60.0% solvent; large and small dashed lines = pure solvent).

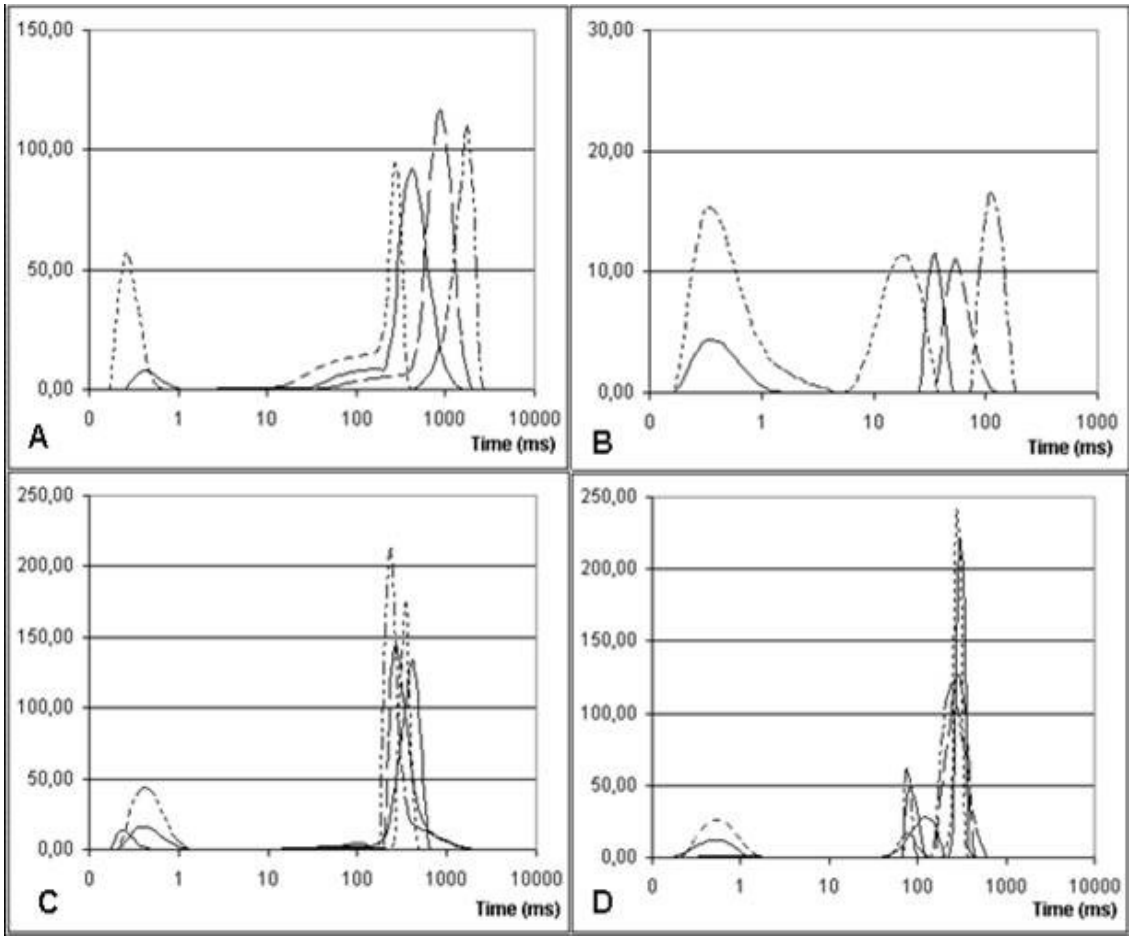
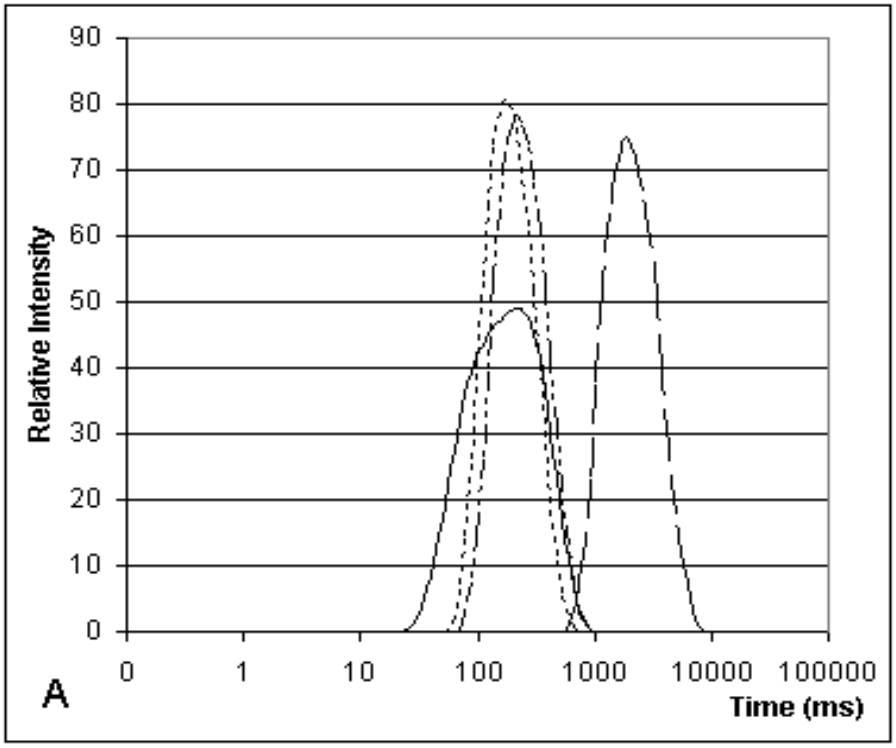
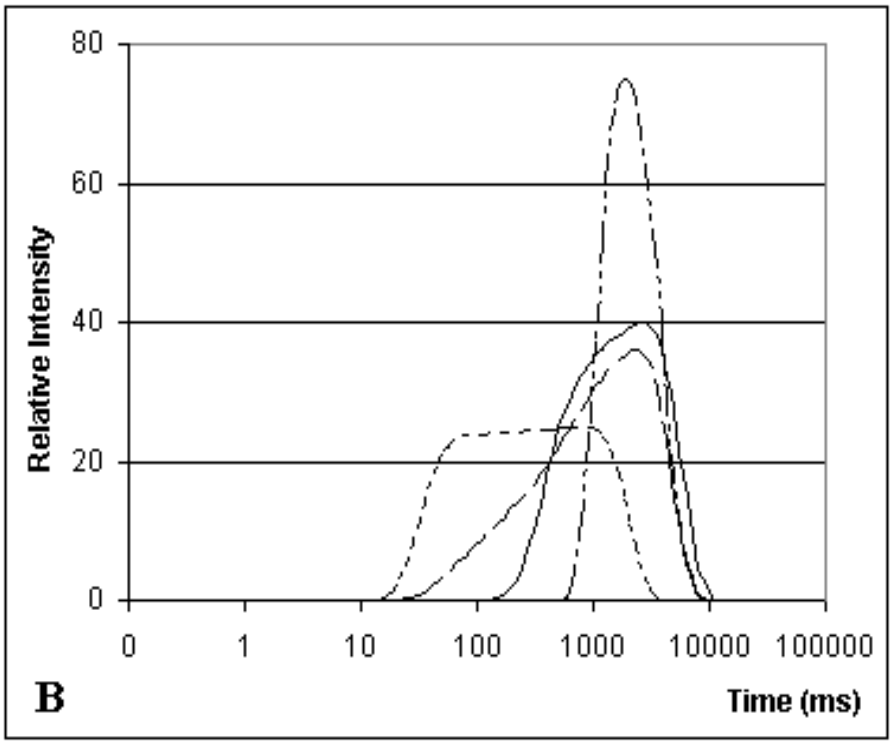


Figure 6A. $^{1}H$ $T_1$ curves for pure solvents (solid line = MCT; dotted line = propylene glycol; large and small dashed line = triacetin; large dashed line = ethanol).

Figure 6B. $^{1}H$ $T_1$ curves for starch/ethanol mixtures at different ratios (dotted line = 16.7% ethanol; large dashed line = 33.3% ethanol; solid line = 60.0% ethanol; large and small dashed lines = pure ethanol).



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## I-B. Porous starch for flavor delivery in a tomato-based food application

These results have been submitted for publication to Food Quality and Preference (C. Belingheri, A. Ferrillo and E. Vittadini).


### Abstract

The aim of this study was to evaluate the use of porous starch as a flavor carrier in a tomato-based food application. Plating onto porous starch, plating onto maltodextrin and conventional spray drying were compared as techniques to convert a liquid tomato flavor into powder; resistance to heat stress and flavor content over shelf life were measured by sensory and chemical analyses. Resistance to heat of the three types of flavors was not statistically different. Both sensory and chemical analyses showed that the polarity of the solvent used to carry the flavor molecules onto porous starch is a key factor in determining flavor content over time.


### Introduction

Flavors are widely used in the food industry to improve the sensory attributes of food products that have lost the original flavor of the raw materials during the production processes, especially when heat is involved. Flavors are generally liquid blends of molecules in solvents and are often liable to damage when exposed to heat, air, humidity and other factors[1]. For this reason, liquid flavors are generally converted to powder form to gain a longer stability over time and an easier handling, storage and dosage[2].

Different techniques exist for the conversion of liquid flavors into powder flavors. A liquid flavor may be dispersed onto a bulk powder carrier, such as salt or maltodextrin[3], a technique which allows only a low amount of liquid in the mixture and often requires the use of anti-caking agents (such as silicon dioxide). Liquid flavors may also be mixed with carriers and spray dried to obtain a fine free-flowing powder where the flavor is in the microencapsulated form[4]. A spray dried flavor can have a flavor load of 20%

or more, depending on the carrier used. Microencapsulation protects the liquid flavor from the outside environment thus prolonging its shelf life, whereas a simple blended flavor is not protected from oxygen, air, moisture and heat[3].

In between these two techniques lies the use of porous starch, a relatively new carrier for flavors, believed to be able to entrap molecules with a simple plating procedure[5,6]. Porous starch is a native corn starch that is treated enzymatically to obtain a porous "sponge-like" structure with a large surface to volume ratio. It can be used as a carrier for flavors due to its ability to host flavor molecules and solvents inside its porous structure[5]. Previous studies have shown its capability of encapsulating various substances allowing a high load of the liquid flavor[6]; it is however not clear if the porous starch behaves simply like other bulking agents or if its porous nature protects the flavor as a microencapsulating structure would. The advantages of using porous starch would be mainly the lower production costs (simple plating rather than spray drying) and the high liquid to powder ratio achievable (even higher than in spray drying).

The present study aimed at evaluating the protection from heat and during storage that the porous starch can confer to a tomato flavor carried onto it, compared to a flavor encapsulated by spray drying and a flavor blended onto a non-porous carrier (maltodextrin).

A liquid tomato flavor was converted to powder by either spray drying or plating onto maltodextrin and porous starch. The three flavors were then applied into a finished food product, a commercially available tomato sauce, and evaluated by sensory analysis after sterilization and by sensory and chemical analysis after ageing under real shelf life conditions for six months.

## Materials and Methods

*Preparation of powder flavors*

A tomato flavor (Kerry Ingredients and Flavors, Italy) was converted into powder using three different methods:

- Spray drying: the flavor was dissolved into Medium Chain Triglycerides (MCT, 99.7%, Nutrivis Srl) and a slurry was produced using Gum Arabic (Kerry Ingredients UK Ltd) and maltodextrin (DE 20 potato maltodextrin; Brenntag SPA) as carriers at a 1:3 ratio, obtaining a slurry at

40% solids. The slurry was fed to a single stage spray dryer (APV, Italy; $T_{in}$ = 160°C; $T_{out}$ = 90°C).

- Plating onto porous starch: porous starch (StarrierR®, Cargill) was blended by hand in a 1:1 ratio with the liquid flavor, which had been previously diluted into an appropriate solvent out of propylene glycol (99.8%, Univar SPA), triacetin (99.0%, Chemical SPA) and MCT.

- Plating onto maltodextrin: the same procedure was used to blend the flavor onto maltodextrin however the flavor was diluted with MCT and the powder:liquid ratio was 2:1.

*Preparation of flavored tomato sauce*

All powders had the same flavor fraction content and were thus equally dosed into an industrially prepared unflavored tomato sauce (Santa Rosa Classica sapore crudo, Italy), at a 0.03% level. The sauce was heated to 50°C, and the flavor was then added and stirred until complete dissolution. Sauces containing the spray dried flavor, the flavor plated onto maltodextrin and the flavor plated onto porous starch were labeled SD, PM and PPS respectively. For the flavor plated onto porous starch, the subscripts PG, TA and MO were used to identify the solvent present in the flavor, for propylene glycol, triacetin and MCT respectively.

*Preparation of sterilized flavored tomato sauce*

The flavored sauces were weighed (250g) into retortable glass jars (250ml; Quattro Stagioni, Bormioli Rocco, Italy) and sterilized in a retort (Levati Food Tech, Parma, Italy) using the temperature cycle outlined in Table 1. Sterilized sauces were stored at room temperature for two days until tasting. The sterilized sauces containing the three flavors SD, PM and $PPS_{PG}$ were identified with the codes SDst, PMst and PPSst respectively.

*Flavor Shelf life*

The three powder flavors were allowed to age at normal storage conditions in plastic non hermetically sealed containers at room temperature in the dark. After three and six months from production they were once again used to flavor the tomato sauce and were subjected to sensory and chemical analysis as the fresh and sterilized sauces had been.

*Sensory Analysis*

Tests were carried out in appropriate booths for sensory analysis[7]. Each booth was equipped with a computer for data registration and a red light was used to minimize visual influences on the results. Panelists had water and unsalted crackers at their disposal to clean their mouths in between samples. The following tests were performed in separate sessions:

Ranking test: At the time of flavor production and after three and six months of shelf life, a ranking test was performed on the flavored tomato sauces following the ISO methodology[8]. A ranking test was also performed on the three sterilized sauces.

At least 40 untrained panelists were used for each ranking test. For each panelist, samples were assigned random 3-digit numbers and sample order was randomized. Each ranking test was split for the attributes of smell and taste, and a reference was provided (tomato flavor in water). The lowest rank (=1) corresponded to the least intense tomato flavor, whereas the highest rank (=3) corresponded to the most intense. Panelists had the possibility of assigning two or more samples the same rank. Data analysis was based on the sum of ranks obtained by each sample.

Difference from reference test: this test was developed on the basis of the Difference from Control test[9]. This method was used to compare the sterilized sauces with the fresh sauces. For this test, at least 20 untrained panelists were used. Each sterilized sauce sample was compared to its fresh reference, based on a 5 level descriptor scale (no difference, slight difference, average difference, large difference, very large difference). To evaluate the panelist's correct assessment, a sample of fresh sauce (hidden) was also compared to the fresh reference. The setup of this experiment is summarized in Figure 1. Panelists were also asked to assign a level of off-note formation to each sample, also based on a 5 descriptor scale. For data analysis the 5 descriptor scale was converted into a 10 point scale where the 5 original descriptors corresponded to 0.0 (no difference), 2.5 (slight difference), 5.0 (average difference), 7.5 (large difference) and 10.0 (very large difference).

*Statistical Analysis*

All sensory data was collected and elaborated using appropriate software (FIZZ Network Acquisition and Calculation modules version 2.46B, BioSystemes, France). The results of the ranking tests were evaluated using a Friedman Test, whereas ANOVA and a post hoc LSD test were applied to the results of the Difference from control test.

*Chemical Analysis*

Firstly, SPME/GC-MS analysis was performed on the unflavored and flavored tomato sauce, in order to identify the flavor molecules present.

Secondly, a qualitative SPME/GC-FID analysis was performed on the same tomato sauces that were tasted to monitor the flavor molecule content over time.

A vial for SPME was prepared by weighing 2g of salt, 35g of deionized water, 50g of flavored tomato sauce and 50µL of Internal Standard solution (ethyl butyrate, 99.9%, [Frutarom]). The vial was equilibrated for 15 minutes at 30°C in a 400ml water bath under magnetic rotation at 1100rpm, and then a syringe for SPME (DVB/CARBOXEN/PDMS 50/30µm fiber, Supelco) was exposed to the headspace for 40 minutes at the same conditions. The fiber was then injected into a Gas Chromatograph (GC 6890, Agilent) equipped with a DB1 column and a Flame Ionization Detector (splitless mode; injector T = 280°C; T1 = 40°C for 5 minutes; ramp 5°C/min to 240°C; final T = 240°C for 10min; detector T = 300°C).

20 molecules, deriving both from the sauce itself as well as from the added flavor, were chosen to be monitored over time, expressed as relative abundance. The relative abundance was calculated using the area of internal standard present, according to formula (1).

Relative abundance X = Area of molecule X / Area of Internal Standard (1)

**Results and Discussion**

*Initial flavor composition*

The flavor powders obtained from the spray drying and plating processes were dry and free flowing and did not undergo caking over six months of shelf life at room temperature. Though visually similar, the three

types of flavor powders have different physical structures. A spray dried powder hosts the flavor molecules in cavities in the wall of the particles; porous starch hosts the flavor inside the pores of the structure but there is no complete block with respect to the outside environment; maltodextrin, finally does not form capsules and does not have a porous structure, so the liquid flavor is simply absorbed onto the surface of the carrier, and it is expected to be the product most susceptible to damage from heat.

The fresh powder flavors SD, PM and $PPS_{PG}$ had the same theoretical flavor content, as described in the materials and methods section, and in the first ranking test performed (Figure 2A) no significant differences were evidenced among the three samples for the attribute of taste. For the attribute of smell, however, SD resulted significantly stronger ($\alpha < 0.05$) than PM and $PPS_{PG}$, possibly due to the dissolution of the spray dried product in the water based tomato sauce resulting in a higher release of volatile molecules into the headspace as perceived by the panelists. Fresh SD also had a higher initial headspace content of certain molecules, as measured by SPME/GC-FID analysis (black bars in Figure 7).

*Effect of heat on flavor intensity*

Tomato sauces flavored with freshly prepared tomato flavor (SD, PM and $PPS_{PG}$) were compared by sensory analysis to sterilized versions of the same sauces (SDst, PMst and PPSst) to verify the protection from heat offered by the different encapsulating methods to the flavor.

Figure 2B shows the results of the ranking test performed on the three sterilized sauces, SDst, PMst and PPSst. Both for the attributes of smell and taste, no significant differences were evidenced among the three samples.

A difference from reference test was also performed comparing the fresh and sterilized sauces, the results of which are shown in Figure 3. For all sauces, the sterilized product had a significantly larger difference from the reference than the fresh sauce, confirming the ability of the judges to determine a difference between the fresh and sterilized sauces. This also means that the sterilized sauce, for each type of flavor, was significantly different in smell and taste with respect to the fresh sauce containing the same flavor. However, no significant difference emerged among the three

different types of flavor. This same consideration holds for the presence of off-notes (results not shown), which resulted, for all types of flavors, significantly higher in the sterilized sauce than in the fresh sauce, but no significant differences emerged between the three types of flavor.

These results indicate that there are, in fact, no differences in behavior of the three forms of powder in protecting the flavor from heat. However, one must consider the strong differences in physical structure existing among the three powders, as discussed earlier. The fact that no significant differences have emerged among the products is, to our best evaluation, to be ascribed to the fact that the powders, once placed in a water-rich environment, lost that physical structure that should protect the flavor during heat treatments.

*Effect of storage on flavor intensity*

Figures 4-6 show the results of the sensory analysis (ranking tests) performed on the sauces flavored with the fresh and aged (3 and 6 months) flavors. The higher the rank attributed, the stronger the tomato flavor was perceived by the panelist. The difference between the three series lies in the solvent used to plate the tomato flavor onto porous starch. Three different solvents were selected because previous results showed a different performance of the porous starch as flavor carrier in presence of different solvents[10]. Propylene glycol is the most polar of the three solvents, MCT is apolar and the polarity of triacetin lies in between.

SPME/GC-FID analysis was performed on the 5 flavored sauces SD, PM, $PPS_{PG}$, $PPS_{TA}$ and $PPS_{MO}$ at the same time, and the results are shown in Figure 7. Dimethyl sulphide (DMS), ethyl acetate, 2-methylfuran and 6-methyl-5-hepten-2-one were already present in the unflavored tomato sauce, as determined by the SPME/GC-MS analysis initially performed on the unflavored sauce, and their relative abundance was constant over time considering the tomato sauce was bought fresh for every test (results not shown). For the sake of figure clarity these molecules and the internal standard, though monitored over time, are not shown in the figure.

Figures 4A and 4B show the results of the ranking test in presence of the solvent propylene glycol. For the attribute of taste, it can be clearly seen that $PPS_{PG}$ scored consistently lower than the other two flavors, SD

and PM, even in the fresh products. Over time there was an accentuation of the differences between the products, with $PPS_{PG}$ resulting significantly less intense than the other products after 6 months of shelf life. For the attribute of smell, the fresh spray dried product resulted significantly more intense than $PPS_{PG}$ and PM at time 0, whereas over time the differences between the products became less important, the three products resulting not significantly different after 6 months of shelf life. At the same time, several molecules (mainly α-pinene, camphene, myrcene, α-terpinene, p-cymene, β-ocymene and γ-terpinene) resulted lower in abundance in fresh $PPS_{PG}$ (orange bars in Figure 7) compared to the other four fresh products, even though the initial theoretical flavor content was the same.

The initial molecule content was similar for the other four fresh products SD, PM, $PPS_{TA}$ and $PPS_{MO}$ (black, red, dark green and dark blue bars in Figure 7, respectively), resulting slightly higher in a few cases (mainly α-pinene, p-cymene and γ-terpinene) for fresh SD (black bars in Figure 7). These small differences were however not perceived by the panelists, as can be seen from Figures 5 and 6 where no differences were found between fresh SD, PM, $PPS_{TA}$ and $PPS_{MO}$.

Figures 5A and 5B show the results of the ranking test in presence of the solvent triacetin. Differently to what was observed when propylene glycol was used as a solvent, $PPS_{TA}$ resulted not significantly different from the other products over the entire shelf life considered, both for the attributes of taste and smell. Furthermore, no significant differences ever occurred between all products considered over this length of shelf life.

Figures 6A and 6B show the results of the ranking test in presence of the solvent MCT. Once again, no significant differences occurred among the products, for both attributes of taste and smell, over the entire shelf life considered. $PPS_{MO}$ received, in certain cases, even higher ranks than the other two products though only a borderline statistical difference was calculated for the attribute of taste after 3 months of shelf life.

It is apparent from these three sets of results that the solvent used to disperse the flavor onto porous starch is a key factor in determining the flavor's performance over shelf life, as confirmed also by SPME/GC-FID analysis. In accordance to sensory test results, the flavor content of $PPS_{PG}$ after 6 months (yellow bars in Figure 7) was greatly reduced, compared to

the other 4 aged products, more markedly for those molecules that also initially resulted lower. The flavor content in $PPS_{PG}$, $PPS_{TA}$ and $PPS_{MO}$ (yellow, light green and light blue bars in Figure 7, respectively) followed a pattern according to solvent polarity. For the majority of molecules, where the content was very low in $PPS_{PG}$, it resulted higher in $PPS_{TA}$ and higher still in $PPS_{MO}$ (see for example α-pinene, myrcene, α-terpinene, p-cymene, o-cymene, β-ocymene, γ-terpinene, estragol and β-ionone). These molecules are, in fact, more apolar than polar in nature, and the more apolar the solvent used, the better PPS performed over time. It can therefore be inferred that in order to maintain the flavor content over time, a solvent of similar polarity to the molecules present should be chosen, for plating onto porous starch. This is in accordance with our previous findings[10] where we observed that polar solvents (ethanol and propylene glycol) ensured the highest flavor retention over time when encapsulating a polar molecule (diacetyl). This means that on knowing the composition of a flavor it should be possible to chose an optimum solvent or mixture of solvents to ensure the highest retention of flavor molecules over time, when using porous starch as a carrier. It is important also that the porous starch resulted, in presence of triacetin and MCT, not significantly different from the spray dried product, making the use of porous starch a valid alternative to the spray drying process for converting liquid flavors to powders.

Over time, the majority of the flavor molecules decreased in all products. SD and PM often showed similar levels for flavor molecules after 6 months (grey and purple bars in Figure 7, respectively), with few exceptions where SD seemed to have almost completely lost a certain molecule (α-pinene, o-cymene and β-ocymene). As stated earlier, it is an unexpected result that the flavor plated onto maltodextrin resulted not significantly different from the spray dried product, considering that maltodextrin generally offers no physical protection to the liquid flavor[3], and not only was this detected in the sensory tests but it was also confirmed by the quantification of the molecule content by chemical analysis over time. A plausible explanation for this phenomenon lies in the occurrence of an interaction between flavor and carbohydrate molecules, which has been previously reported[11,12]. Even though maltodextrin doesn't encapsulate the flavors, it is possible that complexes between the carbohydrate and the

flavor molecules are formed, and this can limit the loss of flavor molecules over time. It is possible that a difference between PM and SD becomes more apparent after longer storage times than those considered in this study. It would be interesting to confirm this hypothesis in the future.

**Conclusions**

This study compared three methods for converting liquid flavors to powders, namely spray drying, plating onto maltodextrin or plating onto porous starch, in terms of protection offered to heat and flavor content over shelf life, as measured both by chemical as well as sensory analyses. This study shows a clear correlation between chemical analysis (quantification of flavor molecules present) and sensory analysis (intensity of flavor perceived by humans). This study also clearly shows the important role of choice of solvent for carrying liquid flavors onto porous starch; the higher the affinity between flavor molecules and solvent, in terms of polarity, the higher the flavor retention over time. The potential application of porous starch as a carrier for flavors is confirmed.

**List of Tables**



| Stage | Temperature |
|---|---|
| Start | 0°C |
| Ramp 1 | Heat to 80°C; hold 5 minutes |
| Ramp 2 | Heat to 120°C; hold for 30 minutes |
| Ramp 3 | Cool to 95°C; hold for 15 minutes |
| Ramp 4 | Cool to 60°C; hold for 15 minutes |
| Finish | Cool to 30°C |

**List of Figures**

Figure 1. Experimental setup of the difference from reference test

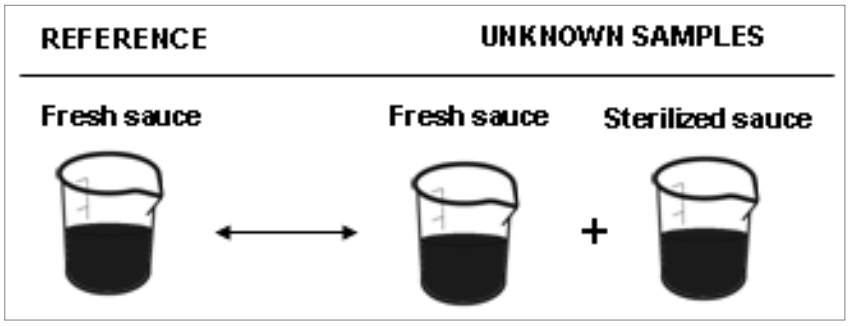



Figure 2. Results of the ranking test performed on fresh (A) and sterilized (B) flavored sauces, for smell (black bars) and taste (grey bars), by 40 untrained judges. SD=spray dried flavor; PM=flavor plated onto maltodextrin; PPS=flavor plated onto porous starch; st=sterilized. A different letter means a significant difference ($\alpha < 0.05$) between samples; capital letters refer to the ranking by smell; small letters refer to the ranking by taste.

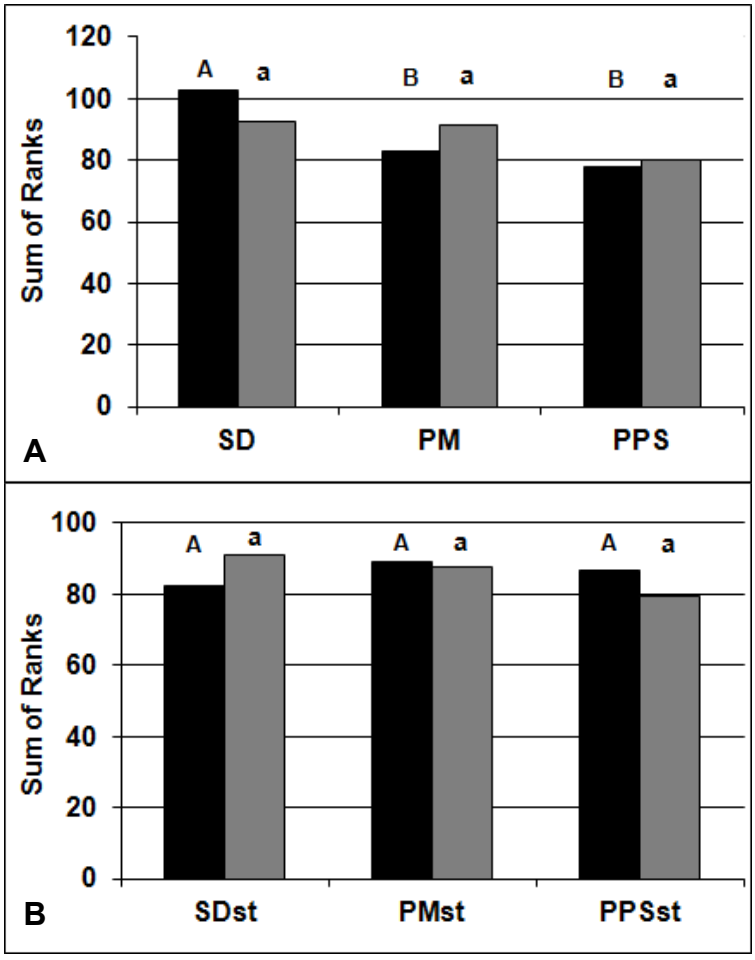


Figure 3. Results of the difference from reference test performed on sterilized flavored sauces using fresh flavored sauces as reference (see Figure 1), for smell (black bars) and taste (grey bars), by 20 untrained judges. SD=spray dried flavor; PM=flavor plated onto maltodextrin; PPS=flavor plated onto porous starch; st=sterilized. A different letter means a significant difference ($\alpha < 0.05$) between samples; capital letters refer to the results for smell; small letters refer to the results for taste.

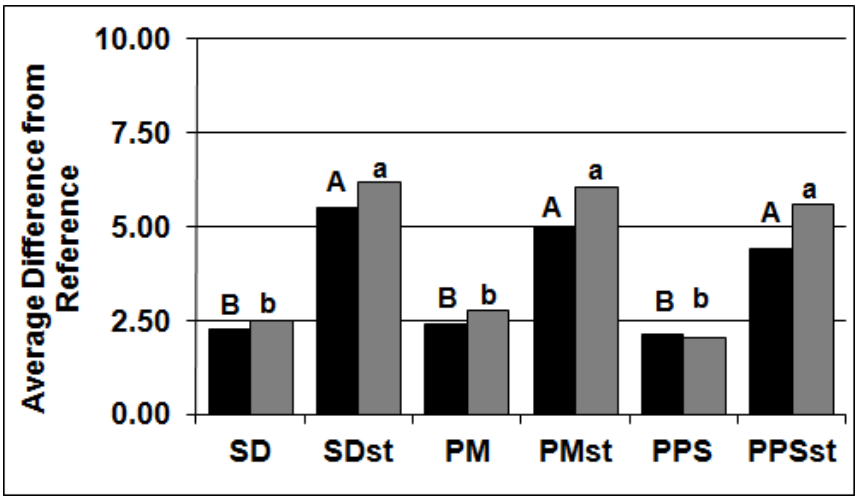



Figure 4. Results of the ranking tests performed on fresh and aged products for taste (A) and smell (B). SD=spray dried flavor; $PPS_{PG}$=flavor plated onto porous starch in presence of propylene glycol; PM=flavor plated onto maltodextrin. A different letter, if present, means a significant difference ($\alpha < 0.05$) among samples.

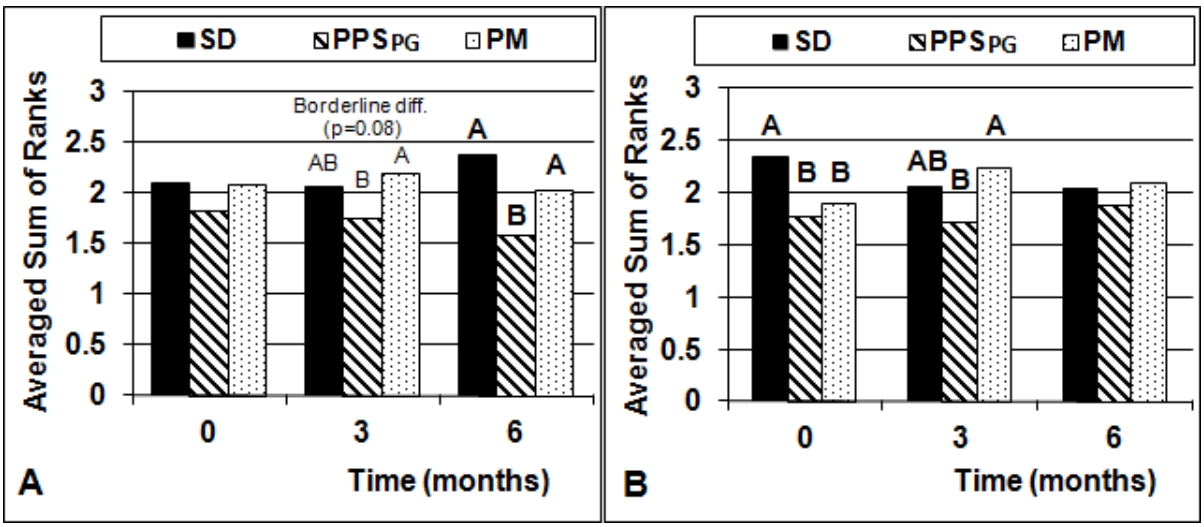


Figure 5. Results of the ranking tests performed on fresh and aged products for taste (A) and smell (B). SD=spray dried flavor; $PPS_{TA}$=flavor plated onto porous starch in presence of triacetin; PM=flavor plated onto maltodextrin. A different letter, if present, means a significant difference ($\alpha < 0.05$) among samples.

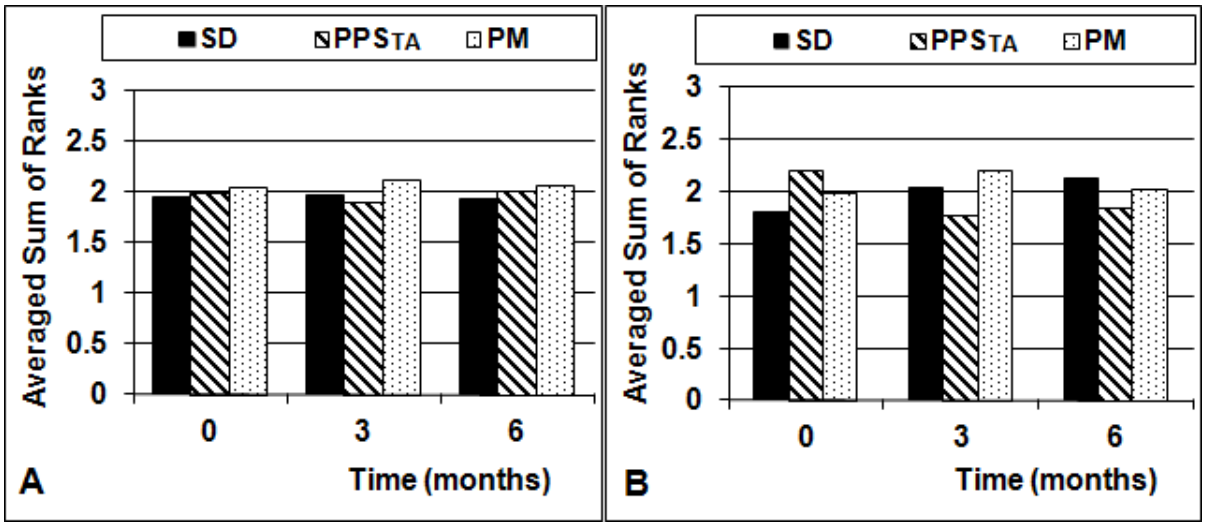



Figure 6. Results of the ranking tests performed on fresh and aged products for taste (A) and smell (B). SD=spray dried flavor; $PPS_{MO}$=flavor plated onto porous starch in presence of MCT; PM=flavor plated onto maltodextrin. A different letter, if present, means a significant difference ($\alpha < 0.05$) among samples.

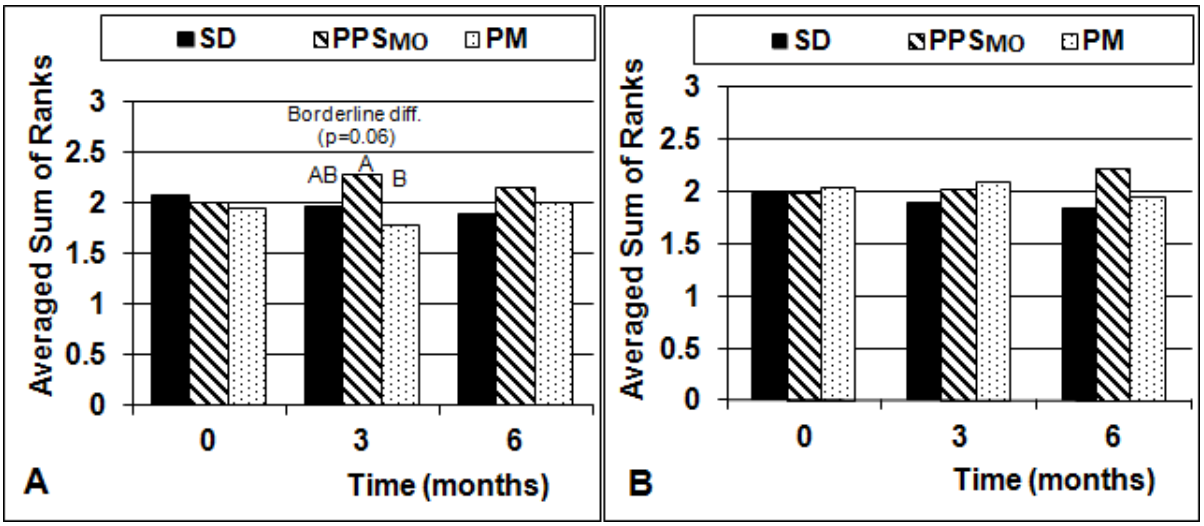
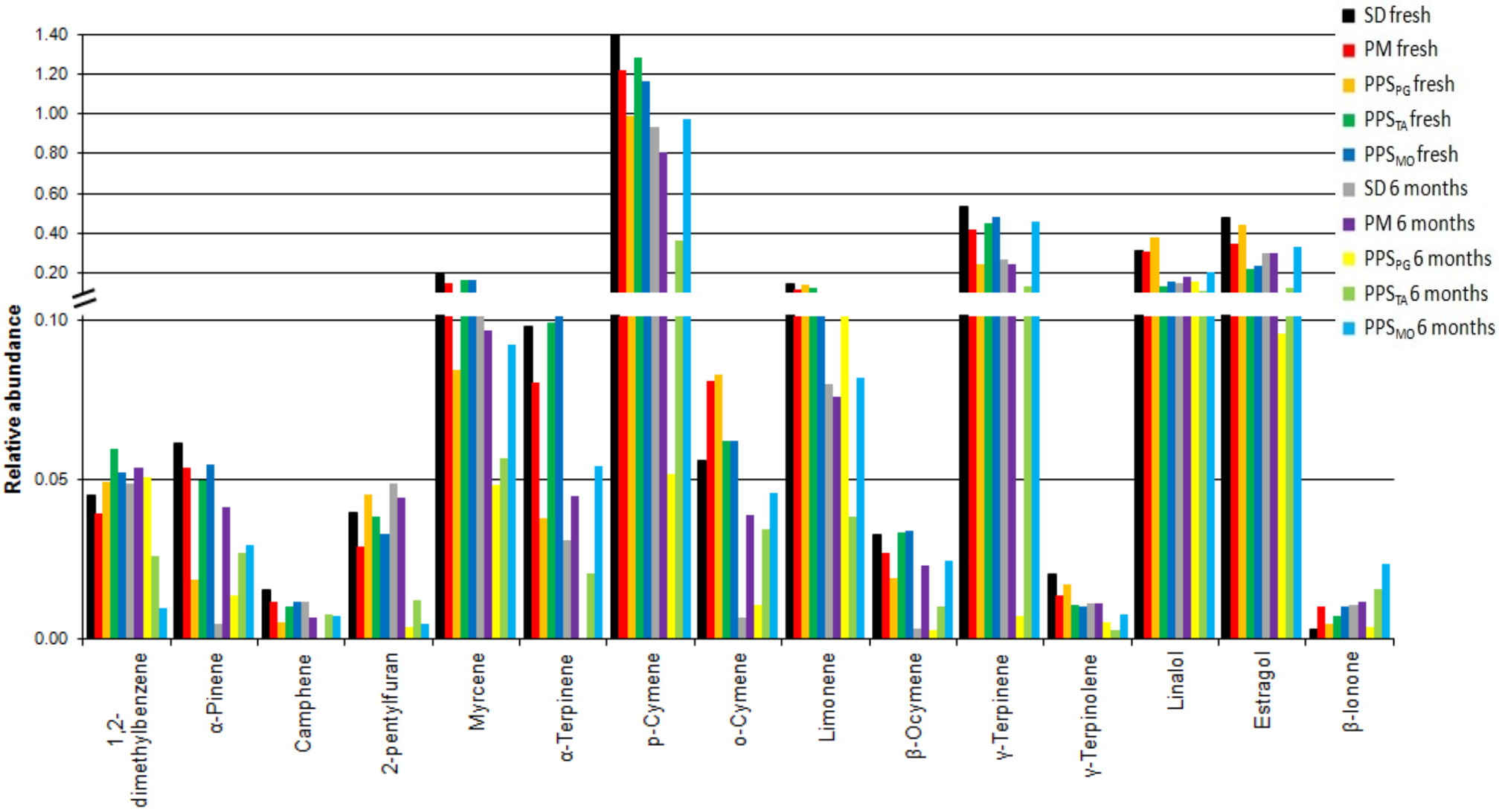


Figure 7. Results of the SPME/GC-FID analysis – relative abundance of molecules in the fresh products and after 6 months of storage. SD=spray dried flavor; PM=flavor plated onto maltodextrin; PPS=flavor plated onto porous starch using as solvent: PG=propylene glycol; TA=triacetin; MO=Medium Chain Triglycerides.



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## I-C. Oxidation of sunflower oil carried on porous starch

These results are to be submitted for publication to Food Chemistry (C. Belingheri, B. Giussani, M. T. Rodriquez Estrada, A. Ferrillo and E. Vittadini).


### Abstract

A design of experiments approach was applied to investigate the effect of microencapsulation by spray drying or plating onto porous starch, on sunflower oil oxidation. Non encapsulated oil, spray dried oil and oil carried on porous starch were stressed by heat and light and the peroxide value and level of conjugated dienes formed were measured. Exposure to light was the most significant factor determining an increase in peroxide value, in all samples. Highest peroxide values were reached by the encapsulated oils, probably because secondary oxidation processes were inhibited and primary oxidation products accumulated. The encapsulation processes determined a reduced effect of light exposure on the increase of conjugated dienes in the oil, compared to the non encapsulated oil. The more significant effect of temperature on the increase of conjugated dienes was also visible from the higher initial level of conjugated dienes in the spray dried oil, which is subject to high temperatures during processing.


### Introduction

All food products and ingredients that contain oils or fats are subject to oxidation, which produces both a sensory deterioration of the product with formation of off notes, as well as a deterioration of its nutritional value, considering the loss of polyunsaturated fatty acids and, in the case of very strong and prolonged oxidation, the formation of toxic compounds[1,2,3].

Lipid oxidation may be better distinguished into autoxidation and photooxidation. In both cases, the initiation of the reaction occurs with the formation or presence of free radicals, and propagates with an autocatalytic mechanism[4]. The primary products of oxidation are hydroperoxides which

then continue reacting to form secondary products such as aldehydes and polar compounds[5].

In the flavor industry, oils are often used as solvents for liquid flavors, because many aroma molecules are oil soluble and also the final applications may be lipid based. The oxidation of such carrier oils would have a negative effect on the overall flavor of the product both because of off note generation from the oil itself, as well as propagation of the oxidation reaction to the flavor molecules, and is thus to be avoided[6]. To limit the oxidative deterioration of oils and sensitive ingredients the industry uses antioxidants, molecules which are very susceptible to oxidation and thus react before the fat components; it is also possible to protect flavors and the carrier solvent from oxidation through microencapsulation, which is the coating of a material (in this case the liquid flavor) with a solid outer wall made of another material[7]. Microencapsulation protects the flavor from the outside environment, so from air, oxygen, light, heat and other components of food that could react with the flavor molecules, thus lengthening the flavor's shelf life[8].

The choice of carrier oil is important in determining the oxidative deterioration of the flavor. It is for this reason that Medium Chain Triglycerides (MCTs) are often used as solvent in the flavor industry. MCTs are a mixture of triglycerides of vegetable origin containing mainly saturated C8 and C10 fatty acids[9]. Saturated fatty acids are much less prone to oxidation because they do not contain double bonds that are most susceptible to attack by free radicals.

Over recent years, however, there has been a growing interest in the food industry to shift to the use of other oils. MCTs are generally derived from coconut or palm kernel oil[10,11], and for the latter there are growing ethical concerns about the negative effect on the environment that cultivation of palm crops has, in terms of deforestation and climate change, traceability and sustainability. Many companies are trying to obtain the totality of their palm oil from certified sustainable sources, while others are trying to substitute their palm oil altogether[12].

A possible substitute to the use of palm oil or palm derivatives is sunflower oil, which is relatively neutral in taste and readily available. The presence of unsaturated fatty acids (80-100%, see Table 1), however,

makes sunflower oil more prone to oxidation than MCTs. Species of sunflower that naturally produce oils with a lower content of linoleic acid exist (high oleic sunflower oil)[10], and these are naturally more stable to oxidation than high linoleic sunflower oil.

The aim of the present study was to evaluate the extent of oxidation of high oleic sunflower oil, and to evaluate the protection from oxidation achieved by conventional microencapsulation (spray drying) or by using a porous starch based carrier, which has recently shown promising potential as a flavor carrier[13].

## Materials and Methods

*Materials*

High Oleic Sunflower Oil was purchased from AarhusKarlshamn, Sweden; porous starch (StarrierR®) was purchased from Cargill; gum arabic was purchased from Kerry Ingredients UK Ltd; DE 20 Potato Maltodextrin was purchased from Brenntag SPA. All other reagents were purchased from Sigma-Aldrich, Germany, unless stated otherwise.

*Sample preparation*

Non encapsulated sunflower oil samples were prepared by pouring the high oleic sunflower oil into 30ml glass bottles that were closed with a plastic screw on cap.

Conventional microencapsulated sunflower oil was obtained by spray drying. High oleic sunflower oil was emulsified with gum arabic and maltodextrin in a 1:3 ratio, obtaining a slurry at 40% solids and a final oil load of 20%. The slurry was fed to a single stage spray dryer (APV, Italy; $T_{in}$ = 160°C; $T_{out}$ = 90°C), the powder was collected and divided into 60ml glass bottles.

Sunflower oil carried on porous starch was obtained by blending the high oleic sunflower oil by hand onto porous starch in order to have a final load of 20%. The powder thus obtained was also divided into 60ml glass bottles.

All samples were prepared in order to have the same surface to air volume ratio of 0.6$cm^{-1}$.

*Design of Experiments*

A two factor Face-Centered Central Composite Design was used in this study, using the variables of temperature and exposure to light as independent variables and peroxide value and level of conjugated dienes as responses (an average of five instrumental replicates was used). Temperature was investigated at 25°C (-1), 32.5°C (0) and 40°C (+1) levels in order to test a "room temperature" situation as well as a less favorable heat stress situation. The three levels of exposure to light tested were 0klux (-1), 300klux (0) and 600klux (+1), values chosen based on preliminary data (not shown). The setup of this experimental design is shown in Figure 1.

Experiments were carried out in a pharmaceutical stability chamber (Pharma Safe System PSC022, SANYO), equipped with white fluorescent lights (Philips 16W Colour 84 HF), which was operated at 6klux/h at the temperatures defined by the experimental design.

Multiple linear regression (MLR) was employed to evaluate the relationships between the independent variables and the response: main, interaction and quadratic effects were taken into account. The regression significance was tested by comparing the effect or variability caused by the regression model to the overall error ($\alpha = 0.05$; significant models have $p<0.05$). All the models here presented have a p-value lower than 0.05.

The goodness of fit of the polynomial models were evaluated by the $R^2$ coefficient and the Lack of Fit test (LOF, $\alpha = 0.05$) for the model found in the ANOVA table. Values of LOF lower than 0.05 indicate that there might be contributions to the variables-response relationships not accounted for by the model. All the presented models have LOF values higher than 0.05.

The modeling was performed using the software The Unscrambler version X 10.2 (CAMO, Norway) and Microsoft Excel Worksheet.

*Oil Extraction from encapsulated powders*

Spray dried powder: oil was extracted from the spray dried powder following a modification of the method for extracting total oil by Bae and Lee[14]. 5g of sample were weighed into a conical bottomed plastic container together with 25g of deionized water and vortexed until complete dissolution. The solution was transferred to a 250ml conical flask and 100g

hexane:isopropanol 3:1 were added. The mixture was stirred with a magnetic stirring bar for 15 min and then centrifuged (ALC centrifuge model PK130) at 3000rpm for 2min. The organic phase was collected into a weighed round flask and the extraction of the aqueous phase was repeated with 50g of hexane:isopropanol 3:1. The second organic phase was added to the round flask and the solvent was evaporated under vacuum using a rotary evaporator (LABOROTA 4000, Heidolph) without heating.

Porous starch powder: 15g of sample were weighed into a 250ml conical flask together with 50ml of chloroform. The mixture was stirred with a magnetic stirring bar for 10min and then filtered under vacuum. The powder was collected and the procedure was repeated. The two volumes of chloroform collected were poured into a weighed round flask and the solvent was evaporated under vacuum using a rotary evaporator without heating.

*Oxidation markers*

Peroxide Value: this analysis is based on the principle that the peroxides formed during the oil oxidation process are able to oxidize Fe(II) to Fe(III) which in turn, on reaction with $SCN^-$, forms a red complex that absorbs at 500nm[15]. By measuring absorbance at 500nm it is possible to calculate the original amount of peroxides present in solution, according to equation (1).

$$\text{Peroxide Value (meq/Kg)} = [(As - Ab)*1/m] / (M_{Fe} * m_o * 2) \qquad (1)$$

Where: As = Absorbance at 500nm of the sample
Ab = Absorbance at 500nm of the blank
m = slope of the Fe(III) calibration curve
$M_{FE}$ = atomic weight of iron, 55.84
$M_o$ = mass of oil in sample

The solutions were prepared and the analysis was carried out following the procedure described by Shantha and Decker[16].

A solution of $SCN^-$ was prepared by dissolving 30g of ammonium thiocyanate in 100g deionized water.

A calibration curve of Fe(III) was constructed by measuring the absorbance at 500nm of standard 10ml solutions of Fe(III) containing 1, 2, 3, 5, 10, 15, 20, 30 and 40µg of Fe(III) to which 50µL of $SCN^-$ solution had been added. A curve with an $R^2$ value of 0.9899 was obtained. The slope "m" was 0.020.

To prepare the standard Fe(II) solution 0.4g of $BaCl_2 \cdot 2H_2O$ were weighed into a 50ml flask and made up to the mark with deionised water. 0.5g of $FeSO_4 \cdot 2H_2O$ were weighed in a 250ml beaker and 50ml of deionised water were added under agitation. The $BaCl_2 \cdot 2H_2O$ solution was poured into the beaker containing the $FeSO_4 \cdot 2H_2O$ solution under constant agitation. 2ml of HCl 10N were added and the precipitate was filtered off. The Fe(II) solution collected was stored in a dark brown bottle away from light.

To measure the peroxide value of oil samples, approximately 0.02g of oil, 9.8ml of chloroform:methanol 2:1 solution and 50µL of $SCN^-$ solution were weighed into a 12ml vial and vortexed briefly. 50µL of Fe(II) solution were then added and the vial was once again vortexed and absorbance at 500nm was measured. A blank vial was prepared by weighing all components except oil, to evaluate the stability of the Fe(II) solution. All samples were read six times.

Conjugated Dienes (CD or $K_{232}$): this value was measured according to the method described in the EUR REG No 2568/91[17]. Approximately 0.1g of oil were weighed into a 10ml flask and made up to the mark with spectrophotometrically pure iso-octane. Absorbance at 232nm was measured with a spectrophotometer (Hewlett Packard Diode Array Spectrophotometer 8452A equipped with HP89532A general scanning software). Pure iso-octane was used as a blank. $K_{232}$ values were calculated according to equation (2).

$$K_\lambda = \varepsilon_\lambda / c * s \qquad (2)$$

Where: $\varepsilon_\lambda$ = Measured absorbance at wavelength λ

c = concentration of sample in g/100ml

s = cuvette width in cm

**Results and Discussion**

The high oleic sunflower oil used in this study was characterized for its oxidation level (peroxide value and conjugated dienes) and the results are summarized in Table 1 together with the fatty acid composition as declared by the producer. The oil showed a low level of initial oxidation, in accordance to quality parameters defined by the Codex Alimentarius[18], with a peroxide value of 3.46 ± 0.12 meq$O_2$/Kg and conjugated dienes content of 2.00 ± 0.01.

*Peroxide Value*

Figures 2A, B and C show the response surfaces for the peroxide values, over the experimental domain considered, for non encapsulated oil, spray dried oil and oil carried on porous starch respectively.

The equation for the significant terms ($p<0.05$) modeling the peroxide value response for non encapsulated oil (Figure 2A) is shown below:

$$Y = 14.95 + 1.41*X_1 + 5.64*X_2 - 4.78*X_2^2 \quad (3)$$

where $X_1$ is temperature and $X_2$ is exposure to light. The model fit the data with an $R^2$ value of 0.95. Exposure to light ($p=8.97e^{-06}$) was more significant than temperature ($p=0.04$) to explain the increase in peroxide value, and the interaction light exposure*light exposure was also significant ($p=3.41e^{-04}$). It can be seen, in fact, that at a fixed value of exposure to light, there is only a small increase in peroxide value going from low to high temperature (for example, at 600 Klux of light exposure, the peroxide value of the oil went from 14.34 meq$O_2$/Kg at 25°C to 17.09 meq$O_2$/Kg at 40°C) whereas at a fixed value of temperature, a much higher increase in peroxide value is seen over the domain of light exposure evaluated (for example, at 25°C, the peroxide value went from 3.56 meq$O_2$/Kg at 0 Klux of light exposure to 14.34 meq$O_2$/Kg at 600 Klux of light exposure). The quadratic effect of light exposure also means that with an increase in light exposure, the increase in peroxide value is not linear. It can in fact be seen that between 300 and 600 Klux of light exposure, at any temperature studied, the peroxide value of the oil reaches a maximum (around 18

meq$O_2$/Kg) and then starts to decrease, possibly indicating that this light stress is sufficient to induce secondary oxidation processes in the oil[19].

The response surface for the peroxide value of the spray dried oil, shown in Figure 2B, fits the data with an $R^2$ value of 0.83 and was linear, both in terms of temperature as well as in terms of light exposure. The equation of the significant terms is shown below:

$$Y = 16.52 + 3.91*X_1 + 8.59*X_2 \quad (4)$$

where $X_1$ is temperature and $X_2$ is exposure to light. As was the case for the non encapsulated oil, exposure to light ($p=2.12e^{-04}$) was more important than temperature ($p=0.02$) to explain the increase in peroxide value. At any fixed temperature, the increase in peroxide value going from 0 to 600 Klux of light exposure was of 11-22 units of meq$O_2$/Kg, whereas at a fixed level of exposure to light, the temperature increase from 25°C to 40°C caused an increase of only 2-12 units of meq$O_2$/Kg. The highest peroxide value reached by the spray dried oil was 29.71 meq$O_2$/Kg, at the highest temperature and highest light exposure.

For the oil carried on porous starch (Figure 2C) the equation of the significant terms is the following:

$$Y = 18.14 + 3.66*X_1 + 9.68*X_2 - 3.60*X_2^2 \quad (5)$$

where $X_1$ is temperature and $X_2$ is exposure to light. The model fit the data with an $R^2$ value of 0.92 and, similarly to the other samples, exposure to light was the most significant factor ($p=2.80e^{-05}$). The other significant terms are temperature ($p=0.01$) and the quadratic term of light exposure with a borderline p value of $5.5e^{-02}$. Even though a quadratic term is present in the model, over the experimental domain studied, no decrease in peroxide value occurs, and the maximum value of 29.24 meq$O_2$/Kg was reached at the highest temperature and highest light exposure.

These data indicate that exposure to light is the most important factor determining the presence and increase of peroxides in oil, in accordance to previous studies where oil autoxidation and photoxidation processes were studied separately, and photoxidation was found to cause a

larger increase in peroxide value of the oil[20]. One would imagine that encapsulated oil would be somewhat shielded from the light, compared to non encapsulated oil, however surface oil subjected to light exposure is probably sufficient to promote the photoxidative reaction. Presence of surface oil on spray dried powders is in fact very negative for product stability, as has been previously reported[21,22]. The porous starch matrix, moreover, is an "open" structure, where surface pores are highly accessible to light. The highest peroxide value reached, however, was very similar for the spray dried oil (29.71 meq$O_2$/Kg) and the oil carried on porous starch (29.24 meq$O_2$/Kg), possibly indicating a high presence of surface oil on the spray dried product.

The non encapsulated oil shows a very rapid increase in peroxide value also at low levels of light exposure (0 to 300 Klux), and a subsequent decrease that is most probably ascribed to the fact that secondary oxidation is allowed to take over. During this secondary oxidation process, the primary peroxides themselves react further and thus lower levels are found in the oil[19]. Both encapsulated oils, on the other hand, don't show a decrease of peroxide value over the experimental domain studied, indicating that possibly secondary oxidation is inhibited and primary oxidation products are allowed to accumulate. It could be hypothesized, for the spray dried product, that the gum Arabic present in the wall matrix has some form of interaction with the radicals present in the oil[23], thus inhibiting the radicalic cleavage of hydroperoxides. A radical scavenging activity of amino acids present in proteins has also previously been reported[24], and the small fraction of proteins present in gum Arabic could also be contributing to reduce secondary oxidation in the spray dried oil. No such activity has however yet been reported for porous starch, and the fact that oil carried onto it shows a similar oxidation pattern to the spray dried oil certainly deserves attention in the future.

*Conjugated Dienes*

Figures 2D, E and F show the response surfaces for the conjugated dienes, over the experimental domain considered, for non encapsulated oil, spray dried oil and oil carried on porous starch respectively.

The equation for the significant terms ($p<0.05$) modeling the conjugated dienes response for non encapsulated oil (Figure 2D) is shown below:

$$Y = 2.19 + 0.05*X_1 + 0.19*X_2 + 0.06*X_1*X_2 \quad (6)$$

where $X_1$ is temperature and $X_2$ is exposure to light. The model fit the data with an $R^2$ value of 0.92 and showed a significant effect both of temperature ($p=0.05$) and exposure to light ($p=2.40e^{-05}$), as well as the interaction between temperature and light exposure ($p=4.70e^{-02}$). In fact, at the low temperature (25°C), the conjugated dienes increased by 0.25 units from 2.02 to 2.27 when the exposure to light went from 0 to 600 Klux, whereas at the high temperature (40°C), over the same interval of exposure to light, the oil's conjugated dienes increased by 0.49 units, from 1.97 to 2.46. Similarly, we can see that at a low light exposure (0 Klux) the conjugated dienes hardly changed over the temperature range studied, whereas at a high exposure to light (600 Klux), the conjugated dienes increased from 2.27 to 2.46 when going from low to high temperature.

The response surface for conjugated dienes of the spray dried oil is shown in Figure 2E and the equation of significant terms is shown below:

$$Y = 2.46 + 0.14*X_1 + 0.10*X_2 - 0.15*X_1^2 \quad (7)$$

where $X_1$ is temperature and $X_2$ is exposure to light. The model fit the data with an $R^2$ value of 0.93 and had a quadratic pattern. As well as temperature ($p=8.92e^{-05}$) and light exposure ($p=1.1e^{-03}$), the quadratic interaction of temperature was also significant in this model ($p=5.1e^{-04}$). At any given temperature the conjugated dienes increased linearly with increase in light exposure. However, at any given level of light exposure, it can be seen that with an increase in temperature the conjugated dienes first increased and then started to decrease. A maximum value of 2.59 was reached at a temperature of 36°C and highest light exposure (600 Klux). This model shows that for the spray dried oil sample, temperature influences the increase in conjugated dienes more than the exposure to light.

For the oil carried on porous starch (Figure 2F) the equation of the significant terms is the following:

$$Y = 2.19 + 0.1*X_1 + 0.23*X_2 \quad (8)$$

where $X_1$ is temperature and $X_2$ is exposure to light. The model fit the data with an $R^2$ value of 0.90, and the significant factors were temperature ($p=5.24e^{-03}$) and light exposure ($p=1.70e^{-05}$), producing a linear model. The conjugated dienes increased linearly both with light exposure as well as with temperature, producing a maximum value of 2.52 at the condition of highest temperature and exposure to light.

The formation of conjugated dienes in non encapsulated oil was found to be more sensitive to exposure to light than temperature overall, but the combination of these two factors enhanced the oil's degradation. Encapsulation, both by spray drying as well as carrying on porous starch, produced a reduced effect of light exposure on the formation of conjugated dienes, with temperature becoming the main factor causing an increase in these components. It is interesting to notice that the oil plated onto porous starch and the non encapsulated oil contained less conjugated dienes before being stressed ($K_{232}$ value of approx. 2.00 at 0 Klux of light exposure) whereas the spray dried oil had an initial conjugated dienes value of 2.06-2.35 at 0 Klux of light exposure. This may be due to the spray drying process itself, as has been previously reported[25], because during spray drying the oil is subject to high temperatures even though only for few seconds. Porous starch has the advantage of not requiring a heating step in the encapsulation process.

Furthermore, at a temperature of 25°C, oil carried onto porous starch maintained the lowest absolute value of conjugated dienes over the entire domain of light exposure evaluated in this study. The highest absolute value for conjugated dienes (2.59) was reached by the spray dried oil at 36°C and 600 Klux of light exposure.

**Conclusions**

It is evinced from this study that the encapsulation of oil modifies the kinetics of the oxidation process. The values of both parameters measured, peroxide value and conjugated dienes, indicate that non encapsulated oil may be subject to secondary oxidation processes before the encapsulated oils. The quantification of molecular markers for secondary oxidation processes would help confirm this hypothesis.

Similar absolute values of peroxides and conjugated dienes were reached at the highest stress level for spray dried oil and oil carried on porous starch. Both these techniques are applicable for reducing the effect of light exposure on the oil over the experimental domain considered, however the spray drying process itself causes an increase in conjugated dienes in the oil. Plating on porous starch seems to be a valid alternative to spray drying for the encapsulation of sensitive oils as it avoids a heating step that induces a start of oxidation in the oil.

**List of Tables**



| | |
|---|---|
| Fatty Acid Composition | C16:0 – 3-5%<br>C18:0 – 2-5%<br>C18:1 – 77-85%<br>C18:2 – 4-15%<br>C18:3 – 0-1%<br>C22:0 – 0-2% |
| Peroxide Value ($meqO_2/Kg$) | 3.46 ± 0.12 |
| Conjugated Dienes ($K_{232}$) | 2.00 ± 0.01 |

**List of Figures**

Figure 1. Setup of the experimental design (two factor, Face-Centered Central Composite Design).

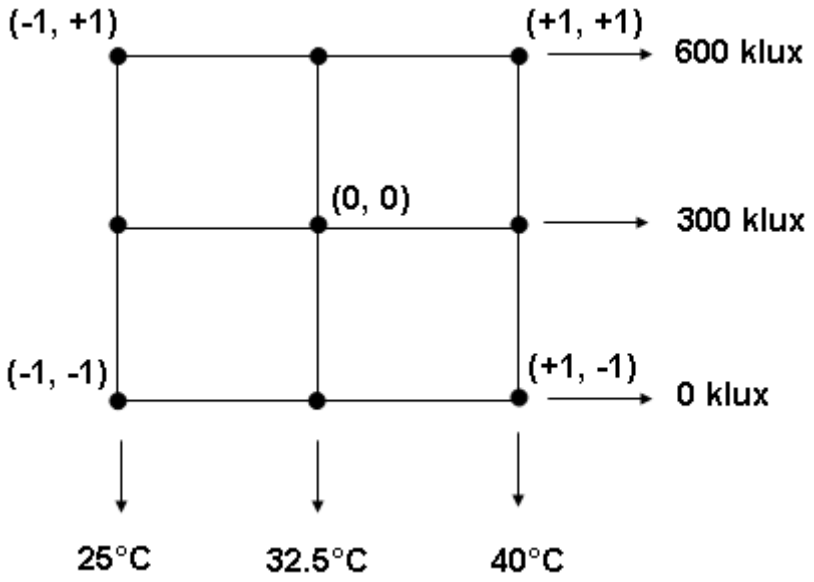
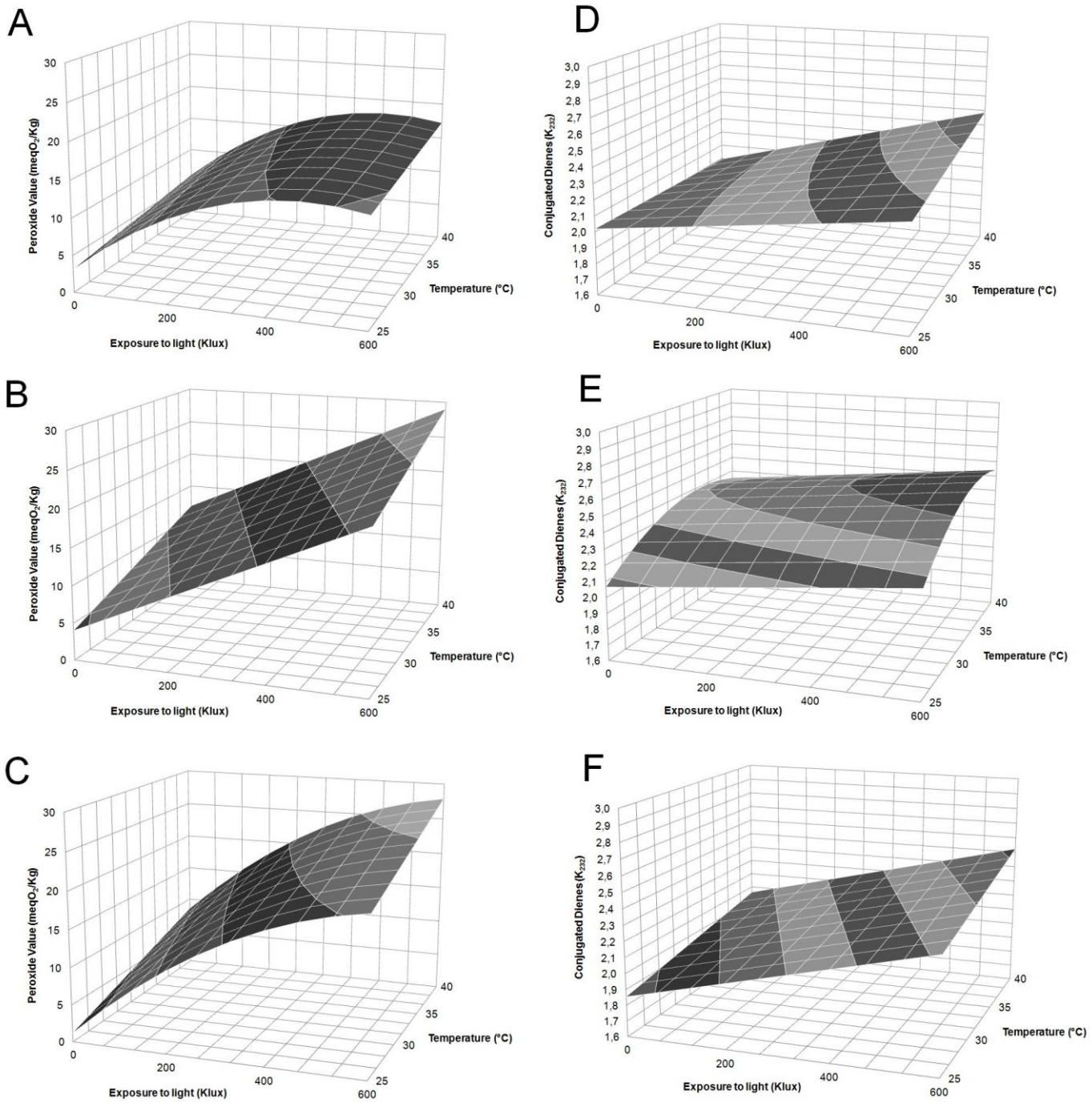


Figure 2. Response surfaces for peroxide values (A-C) and conjugated dienes (D-F). A and D: non encapsulated oil; B and E: spray dried oil; C and F: oil carried onto porous starch.



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# Part II – Comparison of Existing and New Wall Materials for Spray Drying

These results have been submitted for publication to the Journal of Food Engineering (C. Belingheri, A. Ferrillo and E. Vittadini).

**Abstract**

Different wall materials (gum arabic, maltodextrins of potato and pea origin, glucose syrup, modified starches and yeast β-glucans) and their combinations were used for the spray drying of a model molecule, diacetyl, and directly compared in terms of initial encapsulation efficiency and diacetyl retention over time. The range of diacetyl encapsulated was 40-60% of the theoretical total, with the exception of yeast β-glucans which encapsulated only 16% diacetyl. A modified starch was the best performing wall material encapsulating more than 60% diacetyl. Glucose syrup caused lower initial retention of diacetyl in all products where it was used. Pea maltodextrins performed better than potato maltodextrins, but showed a high variability between batches of the same product. The average loss of diacetyl over 18 months of real shelf life was 11%.

**Introduction**

Spray drying is a well established technique for the production of encapsulated powder flavours. Its versatility and relatively low cost have contributed to its widespread use in the flavor industry[1,2].

When spray drying a liquid flavor, the choice of wall material is of great importance. The wall material, or combination of wall materials used, will determine the properties of the finished product, such as dispersibility in water, specific weight, flavor retention and above all, flavor shelf life[3]. A good wall material for flavor spray drying should be inert towards the encapsulated molecules, have a neutral flavor, be highly soluble in water, have a low viscosity in solution to ensure pumpability at high solids, have emulsion stabilizing and film forming properties, and should allow water to evaporate during the drying process while entrapping the flavor molecules[1,4]. Furthermore, once in powder form, the wall material should provide a barrier to oxidation, humidity and temperature to protect the flavor molecules during shelf life[5,6]. Many carriers exist for spray drying, and they are discussed below.

Gum Arabic is historically one of the most popular wall material for spray drying[7]. It is a natural gum composed of a mixture of polysaccharide chains. The backbone structure is a chain of β-D-galactopyranosidic residues joined by β-1,3 linkages with side chains made of the same

residues linked in position 6. Other minor sugars present are L-arabinose, L-rhamnose and D-glucuronic acid[8]. Gum Arabic also contains a small portion of proteins that confer it good emulsion stabilizing properties ("E414" under the class of emulsifiers). It produces relatively low viscosity slurries at high concentrations[9] and is neutral in taste. According to various publications, Gum Arabic has a higher efficiency and advantages of use over other wall materials[10-12]. Gum Arabic has, however, over the past years, shown a high variability in price and availability. This, together with the fact that it is classified as an additive, has lead to the search for different wall materials to replace it[13].

N-Octenyl Succinic Anhydride (n-OSA) modified starches are also widely used for the spray drying of flavors. Starch is derivatized with n-OSA at a level of 3% maximum, which confers good emulsion stabilizing and film forming properties to the starch[14]. Different n-OSA starches exist depending on the degree of n-OSA substitution and of depolymerization which influences starch's viscosity in water and its barrier properties[15]. N-OSA starch is also considered an additive (emulsifier, E1450). In some studies it was found, on the contrary to what was stated by others[10-12], that n-OSA starches have a better encapsulation efficiency than Gum Arabic and other wall materials[16-18].

Another large class of wall materials are starch digestion products, classified based on their Dextrose Equivalent (DE)[5]. The higher the DE, the more the starch has been digested, so the less glucose units will be in each chain. Products with a DE between 2 and 20 are called maltodextrins, whereas products with a DE above 20 are called glucose syrups. Maltodextrins and glucose syrups by themselves are not very good wall materials because they do not have emulsifying properties. They are however widely used in combination with other wall materials, such as Gum Arabic and n-OSA starches because they are highly soluble in water but do not increase the solution's viscosity[1,19]. The main maltodextrins used for spray drying are DE6, 10 and 20 maltodextrins, whereas standard glucose syrup is around 40DE. Contrasting results have been published on the influence of DE on encapsulation efficiency of maltodextrins. According to Wagner and Warthesen[20], a higher DE resulted in an improved retention of spray dried carotenes; Anandaraman and Reineccius[21] found that a higher

DE resulted in the formation of a tighter shell with less surface oil, thus a longer shelf life of the encapsulated oil could be achieved; furthermore, Reineccius[22] found that higher DE products resulted in a better protection to oxidation. This would imply that glucose syrups are better wall materials than maltodextrins, independently of the starch source. In a previous study, however, Bangs and Reineccius[23] stated that flavor retention is inversely related to the DE of the carrier.

Starch, and thus its digestion products, can be of different botanical origin, mainly maize, potato and tapioca. Recently the possibility has been explored to obtain starch and maltodextrins from a new source: pea. A patent by Roquette[24] describes the production of maltodextrins of pea origin that have increased flavor retention with *decreasing* DE value. The use of peas as a source of maltodextrin produces certain advantages, first of all a GMO-free and allergen free product, furthermore the pea is a widespread crop[25]. No data is published, however, comparing encapsulation performance of pea maltodextrins with maltodextrins of other origin.

Yeast cell walls are the last carrier taken into consideration in this study. It is already documented that it is possible to use intact emptied yeast cells for the encapsulation of flavors, by infusion and adsorption[26,27]. Considering the chemical composition of the yeast cell wall, however, it could be hypothesized that the lysed cell walls have some of the characteristics of a good wall material for spray drying. The chemical composition of yeast cell walls has been well described over 50 years ago[28,29]. They are composed mainly of β-glucans (29%), mannan (31%) and protein (13%). β-glucans are polymers of glucose containing β-1,3 linkages, with a highly branched structure. The mannans are associated to the protein fraction. Keeping in mind the characteristics of a good wall material it can be hypothesized that, due to the branched structure of the polysaccharide it will have a low viscosity in solution, and that the presence of proteins will confer it good emulsifying properties. Yeast cell walls are also a very cheap raw material which is readily available.

The aim of the present study was to directly compare different wall materials or combinations of wall materials to be used in spray drying, in order to evaluate new or emerging wall materials. Numerous studies have, over the years, evaluated most wall materials available, however, each

study involves only a limited number of wall materials, and each study uses its own set of variables, recipes, material encapsulated and equipment, making comparison of data difficult[7,10-12,15,17,18,30,31]. In this study, a single method of analysis was used to directly compare both existing and new wall materials. A set of well known carriers was chosen and compared to more recently developed carriers, by focusing on the encapsulation efficiency of a model molecule, diacetyl (2,3-butanedione), which is very volatile and difficult to encapsulate. Wall materials evaluated were: Gum Arabic, by itself and in combination with maltodextrin, two of the most classical options for spray drying; 3 different n-OSA starches of similar viscosity, by themselves but also in combination with Gum Arabic and different DE maltodextrins, a combination that has been shown to have a high encapsulation efficiency[6]; potato maltodextrins of DE10 and DE20 and a glucose syrup of DE38, both alone as well as in combinations with Gum Arabic and modified starches, to see the effect of DE on flavor retention; maltodextrins of pea origin were compared to maltodextrins of potato origin, both for DE10 and DE20 products; a first attempt to use yeast β-glucans for spray drying of flavors was made.

## Materials and Methods

*Samples*

Wall materials used were: Gum Arabic (Kerry Ingredients and Flavours, UK); DE10 and DE20 Potato Maltodextrins (Avebe, Holland); DE10 and DE20 Pea Maltodextrins (Glucidex IT7L and Glucidex IT17L, respectively, Roquette, France); DE38 corn glucose syrup (C*Dry GL, Cargill, Italy); 3 n-OSA starches having similar viscosity in water, namely Cleargum CO 01 (Roquette, France), N-Lok (National Starch) and C*Emcap 12671 (Cargill, Italy); Yeast cell wall β-glucans (Mannomax, Kerry Bioscience, UK).

The wall materials and their combinations used in the production of spray dried samples are summarized in Table 1.

*Spray-drying*

Diacetyl (99.0%, Moellhausen SPA) was dissolved into Medium Chain Triglycerides (MCT, 99.7%, Nutrivis Srl) at 5% level and this was used as a

model flavor for all products. Flavor slurries were produced by mixing the appropriate wall materials in water to obtain 40% solids, and the flavor was added at a level of 6.67% on wet basis. The slurry was homogenized for 15 minutes and then fed to a single stage spray dryer (APV, Italy; $T_{in}$ = 160°C; $T_{out}$ = 90°C). For each recipe, at least three batches were mixed and spray dried independently. Products were then stored in non hermetically sealed plastic containers, in the dark, at room temperature for 18 months.

*Diacetyl content*

The content of diacetyl was measured at the moment of production (fresh products) and after 6, 12 and 18 months of shelf life.

Diacetyl was quantified using the SPME method described by Belingheri et al.[32]. Briefly, 0.5g of sample was weighed into a 12ml glass vial together with 2g of salt, 10g of deionized water and 20µL of Internal Standard solution (ethyl butyrate, 99.9%, [Frutarom]). The vial was equilibrated for 10 minutes at 30°C in a 400ml water bath under magnetic rotation at 1500rpm, and then a syringe for SPME (100µm PDMS fiber, Supelco) was exposed to the headspace for 10 minutes at the same conditions. The fiber was then injected into a Gas Chromatograph equipped with DB1 and DB1701 columns and a Flame Ionization Detector (GC 6890, Agilent; Injector T = 280°C; splitless mode; T1 = 40°C for 3 minutes; ramp 10°C/min to 280°C; final T = 280°C for 5min; detector T = 300°C).

Each sample (individual batch) was analyzed at least in triplicate.

*Statistical analysis*

All fresh products were compared using one way ANOVA and post hoc LSD test ($\alpha<0.05$). Single products were then evaluated over time using one way ANOVA and post hoc LSD test ($\alpha<0.05$). All statistical analyses were performed using SPSS Statistics (IBM, version 19.0.0).

## Results and Discussion

*Characterization of fresh spray dried products*

Initially, the 14 products summarised in Table 1 were spray dried and lead to the production of homogeneous, dry powders. The yeast β-glucans could not be used alone due to the very high viscosity of the resulting

solution and were, in order to spray dry at 40% solids like all other products, mixed with maltodextrin (1:1). Slurries produced only with maltodextrins had a tendency to separate over time and were thus kept under agitation during spray drying.

Table 2 summarizes, listed in increasing order, the initial diacetyl content (percentage of the theoretical total) of all spray dried samples. To facilitate the discussion, Figure 1A-D shows the diacetyl content of the spray dried powders grouped by category, keeping Gum Arabic in all graphs as a reference, showing statistical analysis within each group.

The range of diacetyl content went from 40 to 60% for all products except YST which encapsulated only 16.25 ± 4.56% of the theoretical maximum. The majority of samples considered encapsulated between 40 and 50% of total diacetyl, showing that in fact there is not such a large variability among products as might be expected considering the different nature of the wall materials. Furthermore, the encapsulation efficiency can be considered as good overall, taking into account the high volatility of diacetyl. Similar yields have been reported for very volatile molecules such as esters[33]. The highest diacetyl content was obtained with the product MIXCG (61.14 ± 5.62%), a mix of 3 wall materials (Gum Arabic, maltodextrin and n-OSA starch) confirming that this combination yields good results for flavour retention in spray drying[6].

Figure 1A shows the initial diacetyl content of products spray dried with n-OSA starches of different suppliers, compared to Gum Arabic. The n-OSA starch Cleargum, by itself (CG), encapsulated 57.50 ± 5.55% of the theoretical total diacetyl, resulting not significantly different from MIXCG (Table 2) and significantly higher than the other n-OSA starches evaluated (Figure 1A). The three different n-OSA starches performed significantly differently from each other. N-OSA starches can differ for degree of succinilation and depolymerisation; the three starches considered in this study had similar viscosity in solution, indicating a similar degree of depolymerisation, therefore the differences we found may be attributable to the degree of succinilation that influences emulsion and film forming capacities of the starch, and ultimately the encapsulation efficiency[14]. The diacetyl content of the two starches with significantly lower initial diacetyl content, NL and CE, was not measured over shelf life.

Figure 1B shows the diacetyl content of the pea and potato maltodextrins evaluated, compared to gum Arabic. Gum Arabic itself encapsulated less than 50% of the theoretical diacetyl, and both pea maltodextrins (DE10 and DE20) as well as potato DE10 maltodextrin resulted not significantly different from Gum Arabic. This is strange considering gum Arabic is largely regarded as one of the best wall materials for spray drying. To the author's opinion this particular gum Arabic performed poorly and it is not, in fact, the maltodextrins performing exceptionally well. In general, it can be stated that pea maltodextrins performed better than their potato equivalents, in terms of initial diacetyl content. MD20, in particular, resulted significantly lower than both pea maltodextrins and gum Arabic.

Figure 1C shows the diacetyl content of formulations where DE38 glucose syrup was used in replacement of DE20 maltodextrin. Glucose syrup by itself resulted significantly lower than most other products evaluated, with an initial content of only 41.46 ± 8.89% (together with MD20 maltodextrin, better only than yeast β-glucans, see Table 2). Furthermore, in the two products where it replaced MD20 maltodextrin, it caused a significantly lower retention of diacetyl. The standard product, STD, encapsulated 53.56 ± 6.07% diacetyl, whereas STDGLU only encapsulated 48.55 ± 6.64% diacetyl. The best performing product, MIXCG, encapsulated 61.14 ± 5.62% of diacetyl, whereas on replacement of MD20 maltodextrin with DE38 glucose syrup (MIXGLU) only 50.38 ± 10.13% of diacetyl was encapsulated, with a loss of more than 10%. This data shows that a higher DE results in a lower level of diacetyl retention, in accordance with results by Bangs and Reineccius[23].

Figure 1D shows the diacetyl content of yeast β-glucans, compared to the two standard products (GA and STD). As stated before, YST had the lowest initial diacetyl content and it must also be considered that there were difficulties in obtaining a high solids slurry. Moreover, yeast β-glucans did not have a neutral taste but were rather "yeasty" and also brown in color. All these factors bring us to conclude that, even though theoretically this product could have many characteristics of a good carrier, in practice it is not applicable in the industrial form we evaluated. The shelf life of this product was not followed.

*Shelf life*

Figures 2A-C show the diacetyl content over time of the 11 products followed for 18 months of real shelf life. All products showed a decrease over time in diacetyl content, the majority of which took place in the first six months of shelf life. The average loss of diacetyl over 18 months was 11%.

Cleargum CO 01 was one of the few products that showed no significant decrease in diacetyl content over the first 6 months of shelf life (Figure 2A). This, together with the fact that it showed one of the highest initial diacetyl contents, means that for products that require a shelf life up to 6 months it is a highly recommended wall material. Further on in the shelf life study, however, CG showed high losses of diacetyl, ending with around 20% less diacetyl than the initial content.

Gum Arabic showed the ageing pattern common to most of the products studied: the highest loss of diacetyl took place in the first 6 months of shelf life, after which the product showed only small losses over time that did not produce significant differences. The final loss with respect to initial content was around 15%.

In Figure 2B, shelf lives of maltodextrin based products and Gum Arabic are shown. All products except PMD10 showed the same ageing pattern as Gum Arabic, i.e. a large decrease between 0 and 6 months, then no significant difference until the end of shelf life. PMD10 instead showed a more gradual decrease in diacetyl content over time, and after 6 months of shelf life the product was not significantly different to the fresh product; similarly, after 12 and 18 months it was not significantly different from the 6 month old product. An observation to be made is that the difference between final and initial diacetyl content was less than 10% for all maltodextrins. Another observation worth making is that maltodextrin based products tended to have very high standard deviations for all data points, even exceeding 11%, indicating that there is a low repeatability between batches of the same product. From an industrial point of view this may be even more important than the achievement of a higher diacetyl content.

Figure 2C shows the shelf lives of products where DE38 glucose syrup substituted DE20 maltodextrin. A very heterogeneous scenario is clear. A

low initial diacetyl content did not correlate with worse performances during shelf life. Particularly, the product STDGLU showed one of the lowest losses over time of all products evaluated (only 7% diacetyl lost over 18 months of shelf life) resulting not significantly different from the fresh product even after 12 months of shelf life. MIXCG and MIXGLU both showed the same ageing pattern, with a large decrease (around 10% loss) over the first six months, followed by statistically constant values over the remaining shelf life.

The standard product, STD, showed high losses over time (around 14%) but they were gradually spread over the first year of shelf life, with each data point resulting significantly lower than the previous. This formulation is better than pure GA both in terms of initial diacetyl content, as well as in terms of overall shelf life, and furthermore maltodextrin has a lower cost than Gum Arabic, so this formulation results cheaper, with better performances in the case of diacetyl.

Glucose syrup by itself (GLU) lost 13% of diacetyl over 18 months, and the decrease was spread over the entire shelf life. Considering also the fact that initial encapsulation efficiency was low, we believe it to be an inadequate carrier for volatile molecules such as diacetyl.

**Conclusions**

In this study, we directly compared a high number of well known and novel carriers for spray drying, as well as their combinations. The best performing product is an n-OSA starch, alone but also in combination with gum Arabic and maltodextrin. It is established that yeast β-glucans in the commercial form used are not suitable for spray drying due to a high viscosity in solution and non neutral taste and color. Pea maltodextrins encapsulated more diacetyl than their potato equivalents, and especially pea DE10 maltodextrin performed better over shelf life. It was confirmed that a higher DE results in a lower encapsulation efficiency of volatile molecules. It will be possible in the future to apply the same parameters and analytical methods used in this study to evaluate new carriers and compare them directly to the data here obtained.

**List of Tables**

Table 1. Wall materials and their combinations used for spray drying.

| CODE NAME | WALL MATERIALS AND RATIO |
|---|---|
| **GA** | 100% Gum Arabic |
| **GLU** | 100% C*Dry GL (DE38 glucose syrup) |
| **CG** | 100% Cleargum CO 01 |
| **CE** | 100% C*Emcap 12671 |
| **NL** | 100% N-Lok |
| **MD10** | 100% Potato maltodextrin (DE10) |
| **MD20** | 100% Potato maltodextrin (DE20) |
| **PMD10** | 100% Pea maltodextrin (DE10) |
| **PMD20** | 100% Pea maltodextrin (DE20) |
| **YST** | Mannomax (yeast cell walls) and Potato maltodextrin (DE20); 1:1 ratio |
| **STD** | Gum Arabic and Potato maltodextrin (DE20); 1:3 ratio |
| **STDGLU** | Gum Arabic and C*Dry GL (DE 38 glucose syrup); 1:3 ratio |
| **MIXCG** | Gum Arabic, Potato maltodextrin (DE20) and Cleargum CO 01; 1:2:1 ratio |
| **MIXGLU** | Gum Arabic, C*Dry GL (DE38 glucose syrup) and Cleargum CO 01; 1:2:1 ratio |

Table 2. Initial diacetyl content of fresh spray dried products, expressed as a percentage of the theoretical maximum, placed in increasing order. A different letter means a statistical difference among samples ($\alpha<0.05$).

| CODE NAME | DIACETYL CONTENT (%) |
|---|---|
| **YST** | 16.25±4.56 $^{h}$ |
| **MD20** | 40.32±5.88 $^{g}$ |
| **GLU** | 41.46±8.89 $^{g}$ |
| **CE** | 43.10±4.62 $^{fg}$ |
| **MD10** | 44.59±3.30 $^{efg}$ |
| **PMD10** | 47.57±9.98 $^{def}$ |
| **STDGLU** | 48.55±6.64 $^{de}$ |
| **GA** | 49.07±3.67 $^{cde}$ |
| **PMD20** | 49.85±10.43 $^{cd}$ |
| **MIXGLU** | 50.38±10.13 $^{cd}$ |
| **NL** | 53.29±4.39 $^{c}$ |
| **STD** | 53.56±6.07 $^{bc}$ |
| **CG** | 57.50±5.55 $^{ab}$ |
| **MIXCG** | 61.14±5.62 $^{a}$ |

**List of Figures**

Figure 1. Diacetyl content of fresh spray dried products grouped by class of carriers: A – n-OSA starches compared to Gum Arabic; B – maltodextrins of pea and potato origin compared to Gum Arabic; C – spray dried products where glucose syrup has substituted DE20 potato maltodextrins; D – Yeast cell walls compared to standard formulations. A different letter means a significant difference among data points ($\alpha<0.05$).

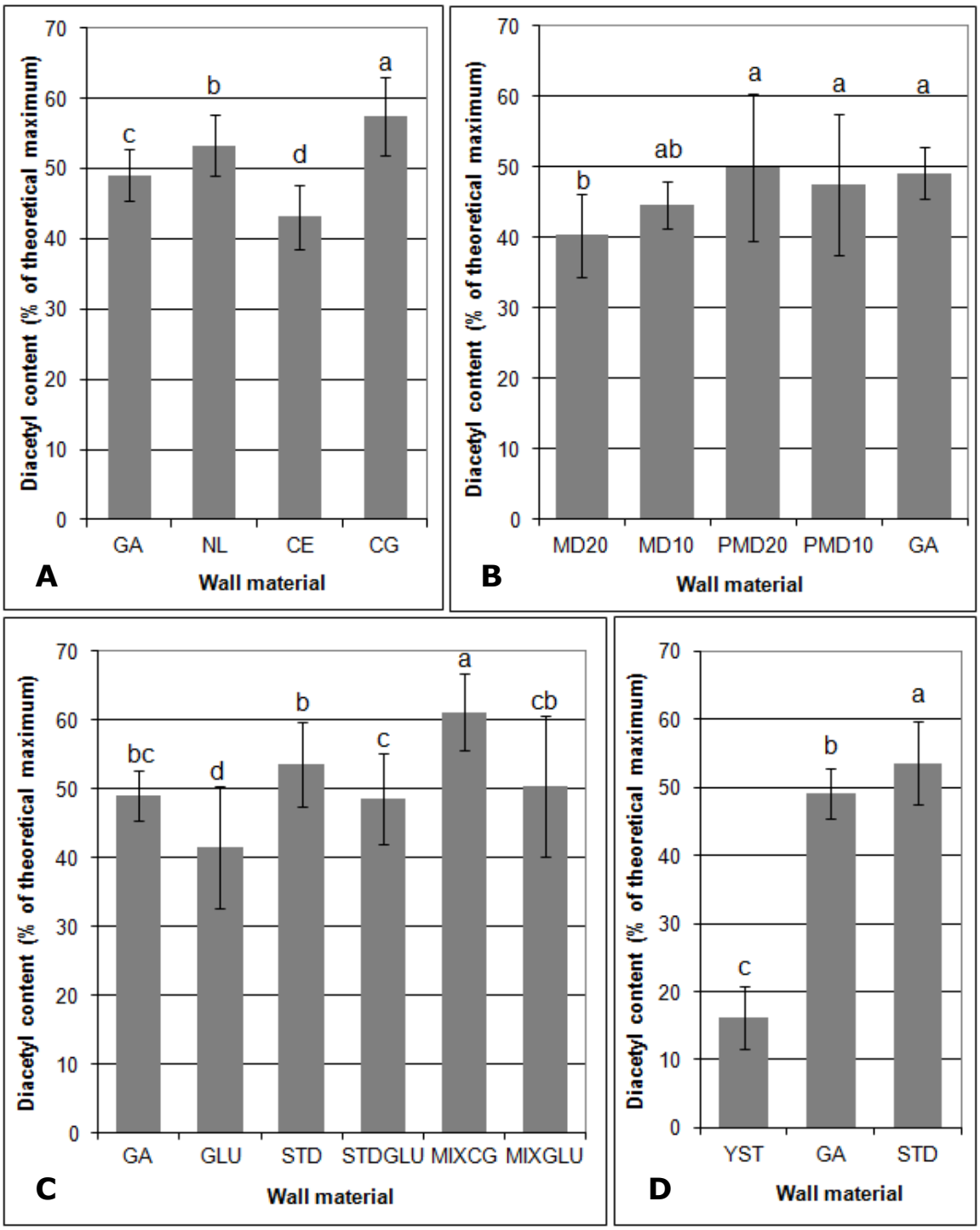
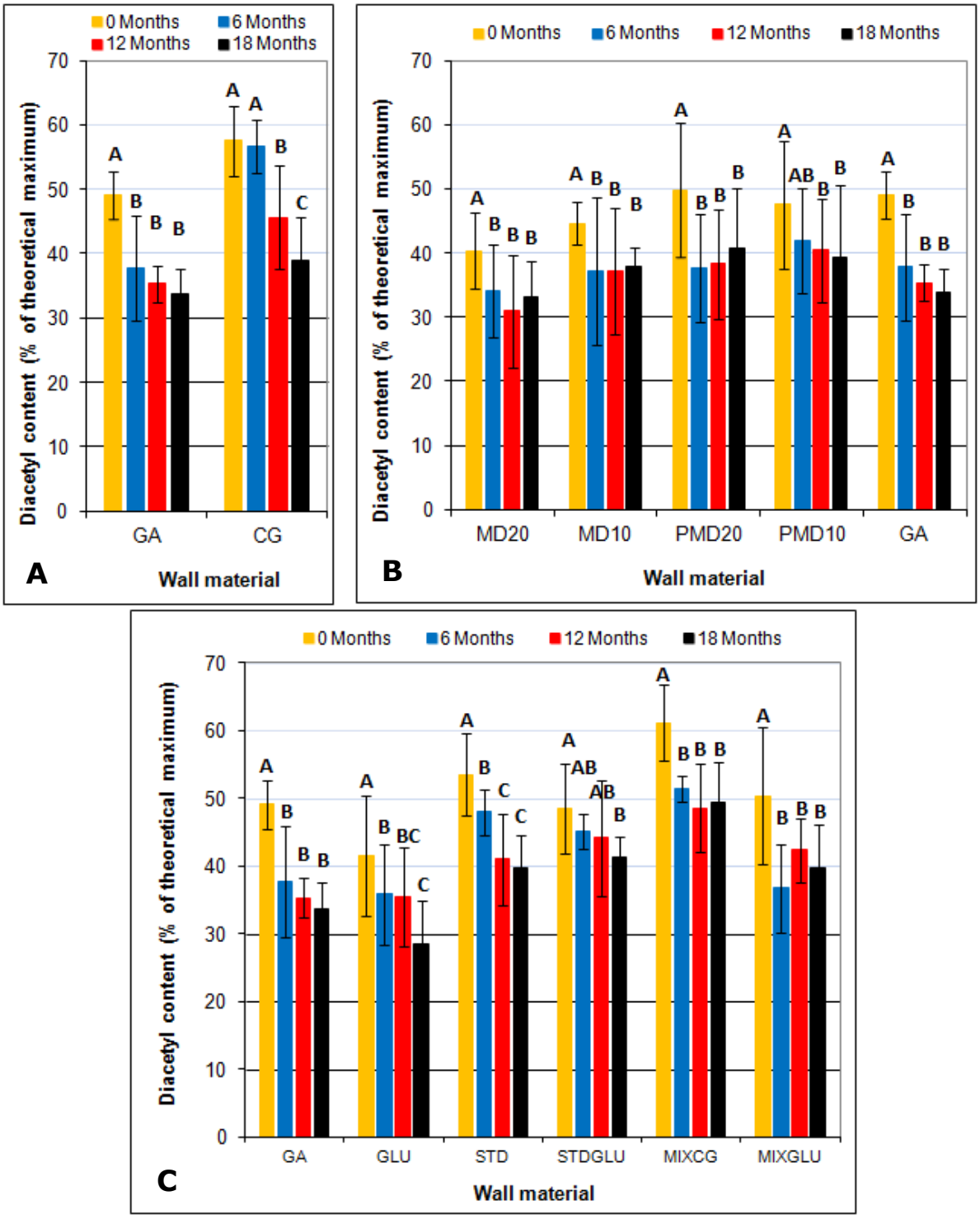


Figure 2. Diacetyl content over time of spray dried products grouped by class of carriers: A – Cleargum CO 01 compared to Gum Arabic; B- maltodextrins of pea and potato origin compared to Gum Arabic; C –spray dried products where glucose syrup has substituted DE20 potato maltodextrins. A different letter means a significant difference over time among data points of each carrier over time ($\alpha<0.05$).



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# Part III – Production of Protein-Carbohydrate Conjugates for Flavor Emulsion Stabilization

**Abstract**

In this section the preliminary studies aimed at developing a scalable method for the production of protein-carbohydrate conjugates are reported. In the first part, the wet state reaction is used and the effect of type of buffer and ionic strength of the buffer on the conjugation reaction is studied through High Performance Size Exclusion Chromatography (HPLC-SEC) and Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE). In the second part an attempt is made to produce nanofibers containing both proteins and carbohydrates, to be used as an alternative to freeze dried powders as substrate for the dry state reaction. These topics are subject of ongoing research of which results will be reported in the future.

**Introduction**

Protein-carbohydrate conjugates are Schiff bases formed between a free amide group on a protein residue and the carbonyl moiety of a reducing sugar present on carbohydrates or in simple sugars[1]. This occurs through the first steps of the Maillard reaction (shown in Figure 1) and, therefore, protein-carbohydrate conjugates are also called Maillard Reaction Products (MRPs).

The conjugation of proteins with carbohydrates enhances many characteristics of proteins. First of all, it gives the proteins a higher emulsifying power, as extensively reported[2-6]. Conjugated proteins also have a higher thermal[7] and pH stability[8] and can show antioxidant properties[9]. All these factors make protein-carbohydrate conjugates of great interest to the food industry in general and, more specifically, the enhanced emulsifying ability makes these products interesting for the flavor industry, where a stable flavor emulsion is very important both for beverage applications[8,10,11] as well as for encapsulation of liquid flavors by spray drying[9,12].

The main drawback to date of MRPs is the difficulty in obtaining high reaction yields and on scaling the reaction industrially, as well as controlling the reaction so as to not proceed beyond the Schiff base formation, thus obtaining undesired secondary reaction products. The two main methods reported to date for the production of protein-carbohydrate conjugates are

the dry state reaction and the wet state reaction. The dry state reaction involves mixing the protein and carbohydrates in a solution which is subsequently freeze dried to obtain a powder where the two reactants are intimately associated; the powder is then reacted at 60°C and 79% relative humidity for a time ranging from 2 to 15 days[8,13-16]. The wet state reaction is usually reported at 60°C in phosphate buffered systems for times ranging from 24 to 72 hours[7,17,18]. Though the Maillard reaction is favored in low $a_w$ conditions[1] the wet state reaction would have the advantages of eliminating a costly freeze drying step and the reaction can be better controlled and limited to the first stages, so to the Schiff base formation[17]. Reported yields are, however, still very low (from less than 5 to about 10%)[17,18], and it is clear that both for the wet and dry state reactions, much still needs to be done before the large scale production of MRPs can be achieved.

As far as the wet state reaction is concerned, which takes place in a buffered system, it is well known that the ionic strength of a buffer and also the type of ions present have a strong influence on the behavior of biopolymers present in solution, and on interactions between biopolymers[19]. Co-solutes, in this case salts, interact both with the water phase as well as with the biopolymers present in solution influencing solubility, protein conformation, protein self-aggregation and thermodynamic compatibility or incompatibility between the polymers[20,21]. Different neutral salt ions influence these properties in different ways, according to their position in the Hofmeister series[22,23] and the ionic strength of the salts in solution[24,25].

The effect of type of buffer and buffer ionic strength on the conjugation reaction in wet state was, therefore, studied, and the results are reported in part A of this chapter.

As far as the dry state reaction is concerned, as stated before, this would be the favored pathway as a low $a_w$ favors the Maillard reaction, but the currently used conditions explained above are not industrially feasible. It was hypothesized that the production of nanofibers containing both protein and carbohydrates would be a convenient substrate for the dry state conjugation reaction, by bringing the two polymers in close contact thus facilitating the conjugation step in a shorter time and with less harsh

conditions. The large scale production of nanofibers is nowadays possible through the needleless electrospinning technique[26].

Needleless electrospinning derives from needle electrospinning, a process which has been well described by Leach et al.[27]. In needle electrospinning a polymer solution is contained in a syringe, whose needle is connected to a power supply; a collector plate is placed some distance away and a potential difference is applied between the needle and the collector plate. The solution, which is slowly pumped out of the needle, becomes charged at the needle tip and is attracted to the collector plate where dry fiber mats are collected (see setup in Figure 2A). With a single needle a very long time is needed to produce usable quantities of fibers. In needleless electrospinning, however, the syringe and needle are substituted by a solution container and spinnerette, of different geometries, that picks up the solution on its surface as it turns. The spinnerette is charged and multiple fiber jets are emitted from the surface of the solution (see Figure 2B) reducing exponentially the time needed for the production of fiber mats. The emission of fibers from the surface of a charged polymer solution was first observed and studied by Yarin and Zussman[28] and more recently needleless electrospinning has been object of various studies using poly(ethyleneoxide)[28,29], polyvinylalcohol[30-32], polyamic acid[33], polystyrene[34] and gelatin[35].

In part B of this chapter we report the preliminary studies on the use of a needleless electrospinning setup, that are the basis for future work on the electrospinning of Dextran - Whey Protein Isolate nanofibers for the large scale production of Dextran - Whey Protein Isolate conjugates.

**List of Figures**

Figure 1. Schiff base formation in the first steps of the Maillard reaction.

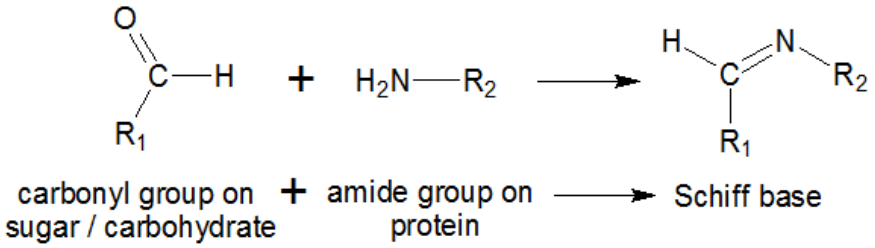
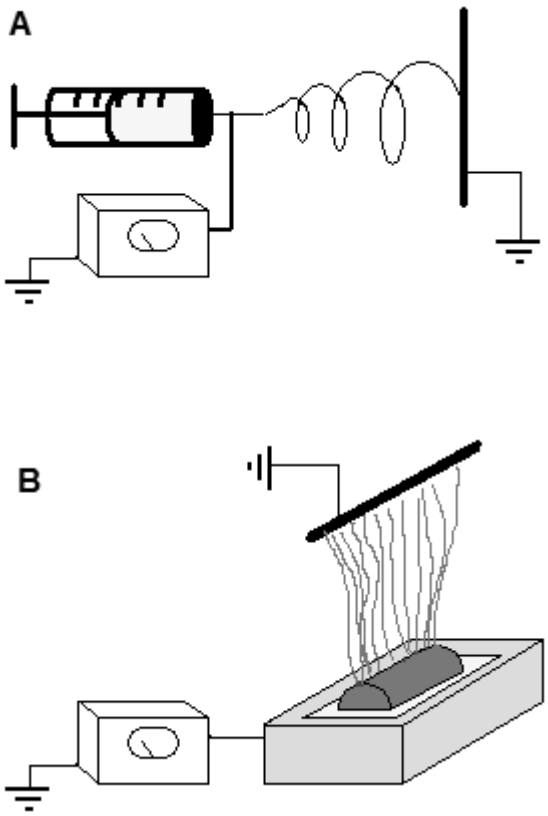



Figure 2. Schematic setup of needle (A) and needleless (B) electrospinning.



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## III-A. Effect of buffer type and ionic strength on the conjugation reaction between Dextran and Whey Protein Isolate

These results are to be submitted for publication to the Journal of Agricultural and Food Chemistry (C. Belingheri, M. Gibis, E. Vittadini and J. Weiss).

### Materials and Methods

*Materials*

Sodium dihydrogen phosphate dihydrate (>99% pure), disodium hydrogen phosphate heptahydrate (>98% pure), citric acid monohydrate (>99.5% pure) and sodium citrate dihydrate (>99% pure) were purchased from Carl Roth and Co. GMBH (Germany). Dextran from *Leuconostoc* spp. (Mw = 40KDa), bovine serum albumin (Mw = 66KDa), egg albumin (Mw = 43KDa), γ-globulin (Mw = 150KDa) and thyroglobulin (Mw = 670KDa) were purchased from Sigma Aldrich (Germany). Whey Protein Isolate (WPI, Lacprodan DI-9224) was a gift from Arla Foods Ingredients (Denmark).

*Conjugation reaction*

The conjugation reaction was performed in presence of citrate buffer or phosphate buffer (same cation, $Na^+$, but different anions) at pH 6.2, with buffer strengths of 10mM, 50mM and 100mM. 10, 50 and 100mM citrate and phosphate buffers were prepared by mixing the appropriate ratio of acid and base, diluting in bidistilled water and adjusting the pH to 6.2 using 0.1M HCl or NaOH.

Reaction solutions of 10% WPI and 30% Dextran were prepared by premixing the powders into the appropriate buffer and leaving them for 8 hours to stir on a magnetic stirrer, after which the solutions were left over night at 4°C to ensure complete hydration and dissolution of the polymers. The solutions were then divided into 1ml aliquots in 1.5ml eppendorf tubes and reacted in a water bath at 60°C for 24 hours following the method described by Zhu et al.[1]. Samples taken for analysis after 2, 4, 8, 12 and 24

hours were immediately cooled to 4°C in an ice water bath and stored at 4°C until analysis.

*Molecular weight determination*

The molecular weights of the reaction products were analyzed by High Performance Size Exclusion Chromatography (HP-SEC) performed on a liquid chromatography system (Hewlett Packard Series 1100 controlled by ChemStation for LC software, version A.08.03, Agilent Technologies) using a tandem of two columns for size exclusion (TSK-Gel 4000$SW_{XL}$ and TSK-Gel 2000$SW_{XL}$, TOSOH Bioscience) preceded by a guard column ($SW_{XL}$, TOSOH Bioscience). A 5mM acetic acid solution containing 0.25M NaCl was used as a mobile phase, at a flow rate of 0.6ml/min. Reacted samples were diluted 20 times with bidistilled water and the sample solution was filtered through a 0.45µm filter before injection into the SEC columns. A volume of 20µl was injected and elution from columns was monitored at 220nm with a Variable Wavelength Detector (Agilent Technologies). The molecular weight of eluted peaks was determined according to a standard molecular weight curve obtained using WPI (MW = 14, 18 and 66KDa for α-lactalbumin, β-lactoglobulin and bovine serum albumin respectively), bovine serum albumin (66KDa), egg albumin (43KDa), γ-globulin (150KDa) and thyroglobulin (670KDa) which produced a logarithmic curve with an $R^2$ value of 0.98 (not shown).

*Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

SDS-PAGE was performed in reducing conditions, according to Laemmli[2], on a Mini-Protean Tetra Cell (Bio-Rad Laboratories) using ready made 15 well precast 10% Tris-HCl gels (Bio-Rad Laboratories). Sample solutions were diluted to 1µg/µl of protein and each well was loaded with 10µl of solution. Electrophoresis was run for 35 minutes at 200V at room temperature. Two identical gels were run at the same time; after electrophoresis one gel was stained for protein using Coomassie blue staining and destained using a 10% acetic acid (v/v) and 15% methanol (v/v) solution and the other gel was stained for glycoproteins using the GelCode Glycoprotein staining kit (Pierce Biotechnology) following the manufacturer's protocol.

## Results and Discussion

*Solution properties*

The appearance of the different solutions before and after reaction is shown in Figure 1. After 12 hours of storage at 4°C and before the conjugation reaction (Figure 1, first row), the solutions in 10mM citrate and phosphate buffers appeared transparent and of low viscosity. With increasing ionic strength the solutions appeared more opaque and viscous, as is observable by the visibility of the magnetic stirrer in Figure 1. No differences were observed between the two buffers at equal ionic strength.

After 2 hours of reaction at 60°C (Figure 1, second row), the appearance of solutions at 10 and 50mM buffer concentration hadn't changed, whereas the solutions at 100mM buffer concentration were visibly more opaque and viscous than the unreacted solutions and no longer poured. Furthermore, on dilution for the HP-SEC analysis, the 100mM citrate solution appeared to be insoluble and was only dispersed under mechanical agitation, while the 100mM phosphate solution readily dissolved in water (Figure 2).

After 4 hours of reaction, no further macroscopic changes had occurred, but it was observed that the 50mM solutions were more difficult to filter through the 0.45µm filter prior to HP-SEC analysis.

After 8 hours of reaction, while the 10mM solutions remained unchanged, the 50mM solutions had also become visibly more viscous than the unreacted solutions and the 50mM citrate solution was less pourable than the 50mM phosphate solution, as can be seen in Figure 1, third row: while the 50mM phosphate solution was still pourable, the 50mM citrate solution did not pour on turning the eppendorf upside down, similarly to the 100mM solutions. These observations didn't change for the remaining reaction time up to 24 hours.

*WPI-Dextran reaction*

Reacted samples were taken after 2, 4, 8, 12 and 24 hours and subjected to HP-SEC (Figure 3) and SDS-PAGE analysis (Figure 4).

The unreacted solutions for all buffers had a single peak in HP-SEC, at an elution volume of approximately 21ml, corresponding to the WPI in solution. For solutions of low ionic strength (10mM citrate and phosphate

buffers) chromatograms showed clearly the time dependent formation of high molecular weight species, above 1000KDa, with a contemporary decrease in the WPI content of the solution (Figure 3A). The behavior of the two solutions was the same, therefore only the chromatogram for 10mM phosphate buffer is shown. In higher ionic strength solutions, however, HP-SEC chromatograms showed a small decrease of the WPI peak over time but no new peaks appeared, even after 24 hours of reaction (for example, 100mM citrate results in Figure 3B).

Gels for SDS-PAGE were loaded with protein molecular weight standards (lane 1), positive and negative controls for the glycoprotein staining protocol (lanes 2 and 3), reaction raw materials (lanes 4 and 5) and a representative set of sample solutions, namely unreacted 10mM citrate and phosphate samples (lanes 6 and 10) and the 24 hour reacted samples for all 6 buffers (lanes 7-9 and 11-13). The positive control for glycoprotein staining appeared in both gels and the negative control was only stained by Coomassie blue, confirming the correct glycoprotein staining of the second gel. The lane containing only dextran (lane 5) was empty as expected, considering dextran is an uncharged polymer and can therefore not migrate into the gel. All the other lanes (WPI raw material and 8 samples) only presented the three bands typical of WPI, namely α-lactalbumin (14KDa), β-lactoglobulin (18KDa) and bovine serum albumin (66KDa) and faint bands for dimers of these components.

*Discussion*

It is apparent from SDS-PAGE analysis that no Dextran – WPI conjugates were formed in the solutions analyzed. The questions that arise are mainly why the reaction hasn’t taken place, what is the identity of the high molecular weight peaks appearing in the HP-SEC chromatograms and how do the differences in solution appearance over time relate to the reaction outcomes.

As stated in the introduction, different interactions are possible between proteins and carbohydrates in solution, and proteins may also interact among themselves. This already complex scenario is further influenced by the presence of salts in solution, depending on the type of salt and the ionic strength.

The first observation made was the increasing viscosity of the solutions at increasing ionic strength. This could be a combination of two different phenomena, namely protein aggregation and salting out. Baussay et al.[3] stated that the aggregation of β-lactoglobulin is influenced by ionic strength, with low ionic strength solutions producing less viscous, more transparent gels with linear aggregates and high ionic strength solutions producing more branched aggregates therefore more viscous and turbid solutions. The aggregation of β-lactoglobulin is promoted at temperatures above 50°C due to monomer denaturation, and formation of irreversible aggregates[4]. It is also reported that at high ionic strengths protein solubility decreases, in a phenomenon known as salting out[5,6], and a specific salting out effect of citrate has also been reported[7]. For the same principle, at low ionic strengths protein solubility is higher. The combination of these two phenomena, aggregation and protein solubility, both dependent on ionic strength, could possibly explain the fact that over time, the soluble aggregates in low ionic strength solutions were visible in HP-SEC chromatograms, whereas the insoluble aggregates formed at high ionic strengths caused the physical almost solid structure of the 100mM solutions, and were probably eliminated from the solutions on filtering before HP-SEC analysis, therefore did not appear in chromatograms.

Considering the fact that in no solution the conjugation reaction was effective, it is possible that the self interaction of protein both in the form of soluble and insoluble aggregates, didn't allow the interaction between proteins and carbohydrates to occur. Furthermore, at high polymer concentrations and high ionic strengths, in presence of two polymers, generally thermodynamic incompatibility occurs[8], which in our case would result in incompatibility between whey protein and dextran with preferential self-interaction of the two polymers. Furthermore, higher protein concentrations promote protein aggregation[9]. A lower overall polymer concentration and a lower ionic strength might favor the interaction between WPI and dextran, though the concentration used in this study was determined as the one producing most Schiff base formation by Zhu et al.[1] who also states that a higher polymer concentration could increase the conjugation reaction yield but it could also result in greater polymerization/aggregation of the protein. The overall polymer

concentration is therefore an issue that still needs to be addressed in the optimization of the conjugation reaction in liquid state.

In the chromatograms for the 10mM solutions (see Figure 3A) the new peak at high molecular weights, that was tentatively attributed to soluble protein aggregates, was clearly increasing over time, representing 60% of proteins present after 24 hours of reaction. This could further confirm the identity of this new peak as soluble aggregates because it is well known that protein aggregation is a time dependent phenomenon also in very complex solutions[10-12].

The second observation was the different behavior between solutions containing citrate and those containing phosphate. Firstly, over the entire reaction time the solubility of the 100mM citrate and phosphate solutions was different (i.e. 100mM citrate reacted solutions were less soluble than 100mM phosphate reacted solutions). Secondly, from 8 hours of reaction onwards, a macroscopic difference was also apparent between 50mM citrate and phosphate solutions with the 50mM citrate solution resulting more viscous and insoluble than the 50mM phosphate solution. In an effort to explain these observations we looked at the Hofmeister series. Well over 100 years ago Hofmeister[13,14] described the different ability of different salts to salt out proteins and produced the now famous Hofmeister series, dividing anions and cations into chaotropic (more salting in or structure breaking effect) and cosmotropic (more salting out or structure forming effect). The order of ions in the Hofmeister series is however not fixed, as the relative order of anions may reverse depending on the charge and hydrophobicity of the interacting surfaces and on the pH of the solution[15,16]. In fact, citrate and phosphate are two anions that lie side by side in the Hofmeister series, and in some cases citrate is reported as having a more stabilizing effect than phosphate[15] whereas in other cases the opposite is reported[7]. In our case it seems evident that in presence of citrate the aggregation of protein is promoted more than in solutions containing phosphate, indicating that citrate has a destabilizing effect on protein structure. This is seen from the insoluble nature of the 100mM citrate solution with respect to the 100mM phosphate solution, and also by the fact that the 50mM citrate solution became more viscous, probably for the presence of branched insoluble aggregates, before the 50mM phosphate

solution which remained pourable even after 24 hours of analysis. In this specific solution, the use of phosphate buffer should be preferred rather than citrate which seems to promote protein self aggregation, and it is desirable to explore further the use of other buffer systems that may delay protein aggregation and promote the interaction between WPI and dextran chains.

The last observation to be made is that in the present study, the 10mM phosphate reaction solution is the same reaction solution used by Zhu et al.[1] who report the successful formation of conjugates even though with a very low yield (around 5%). The only difference lies in the dextran molecular weight, 40KDa in this study compared to 11KDa in the above mentioned study by Zhu et al.[1] Even though they report that the use of a lower molecular weight dextran speeds up the conjugation reaction, a conjugation reaction with higher molecular weight dextran is possible as has been achieved by the same research group using 440KDa dextrans[17]. It is therefore puzzling that in our study, even the 10mM phosphate solution did not produce conjugates. It is possible that the reaction yield was so low that the very minimal amount of conjugates formed was not detectable in our analyses.

**Conclusions**

From the present study it is apparent that the polymer concentration and ionic strength of WPI – dextran solutions are important factors in determining the interactions that occur between the different polymers and, consequentially, the conjugation reaction between the protein and the polysaccharide. More extensive research still needs to be done to increase the wet state conjugation reaction efficiency and the area of salts is in this sense still unexplored and may in the future yield interesting results.

**List of Figures**

Figure 1. Appearance of WPI – Dextran solutions in different buffers before reaction (first row) and after 2 hours (2nd row) and 8 hours (3rd row) of reaction at 60°C.

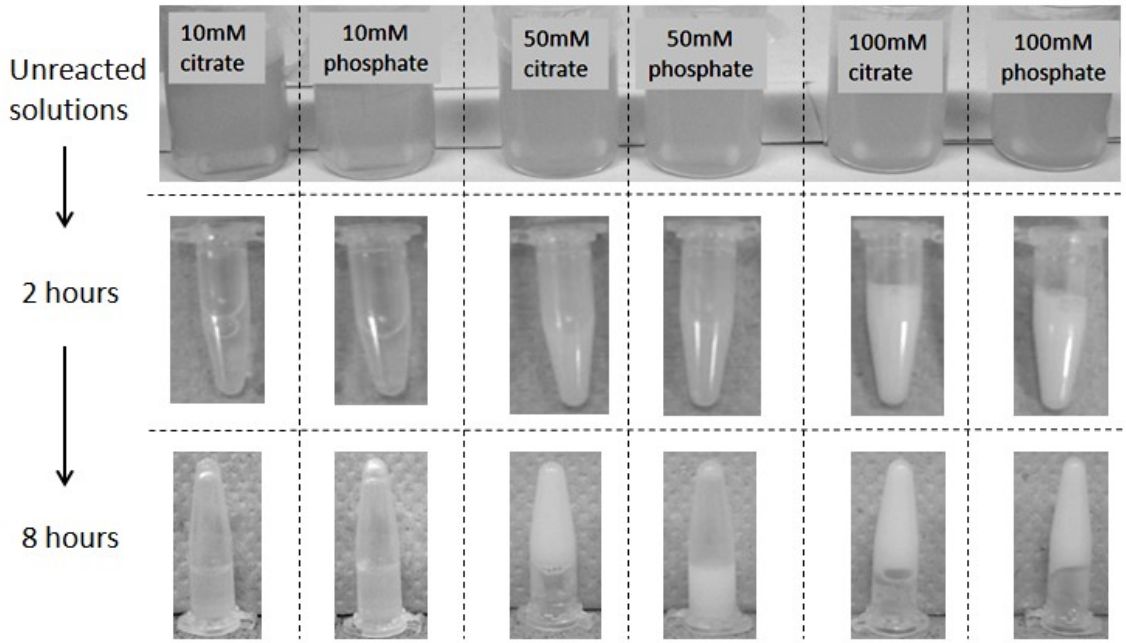



Figure 2. Solubility of WPI – Dextran solutions in 100mM buffers after 2 hours reaction at 60°C.

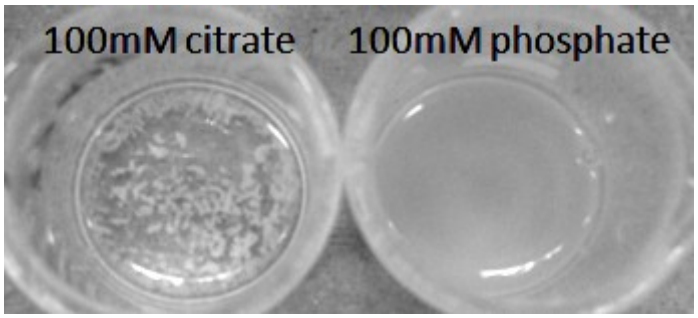
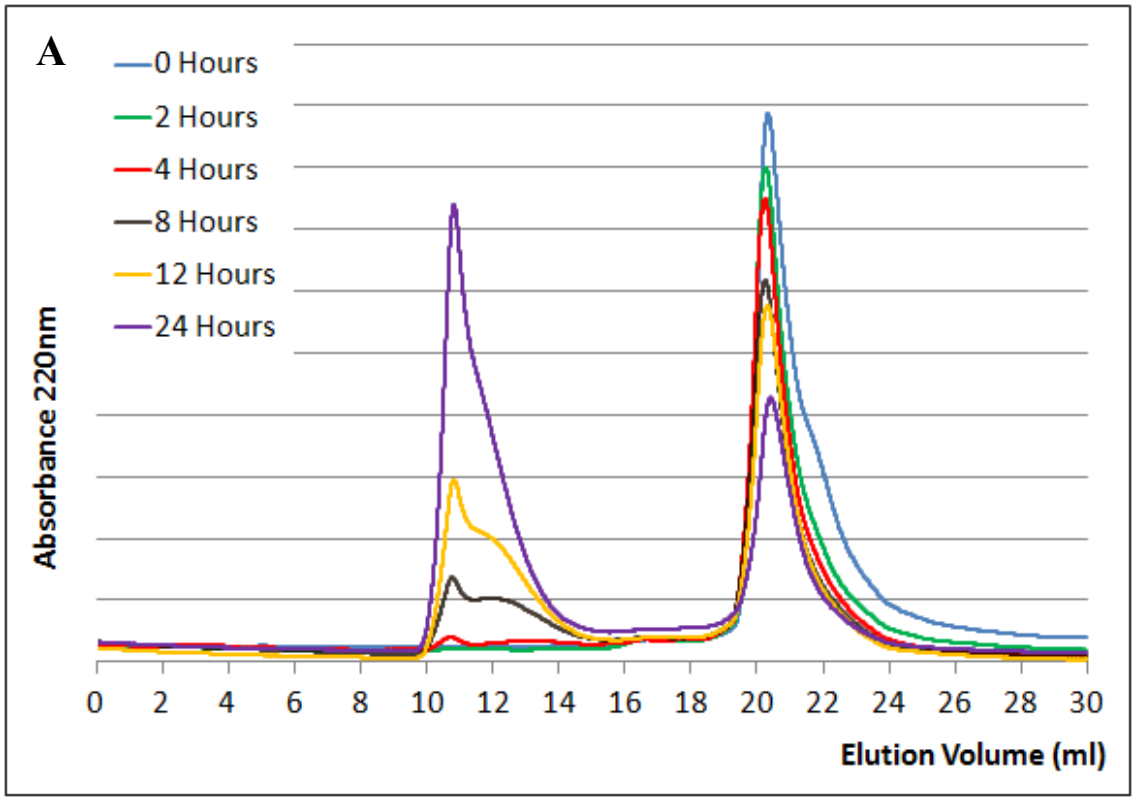


Figure 3. Size Exclusion Chromatography of WPI – Dextran solution in 10mM phosphate buffer (A) and 100mM citrate buffer (B) over time.



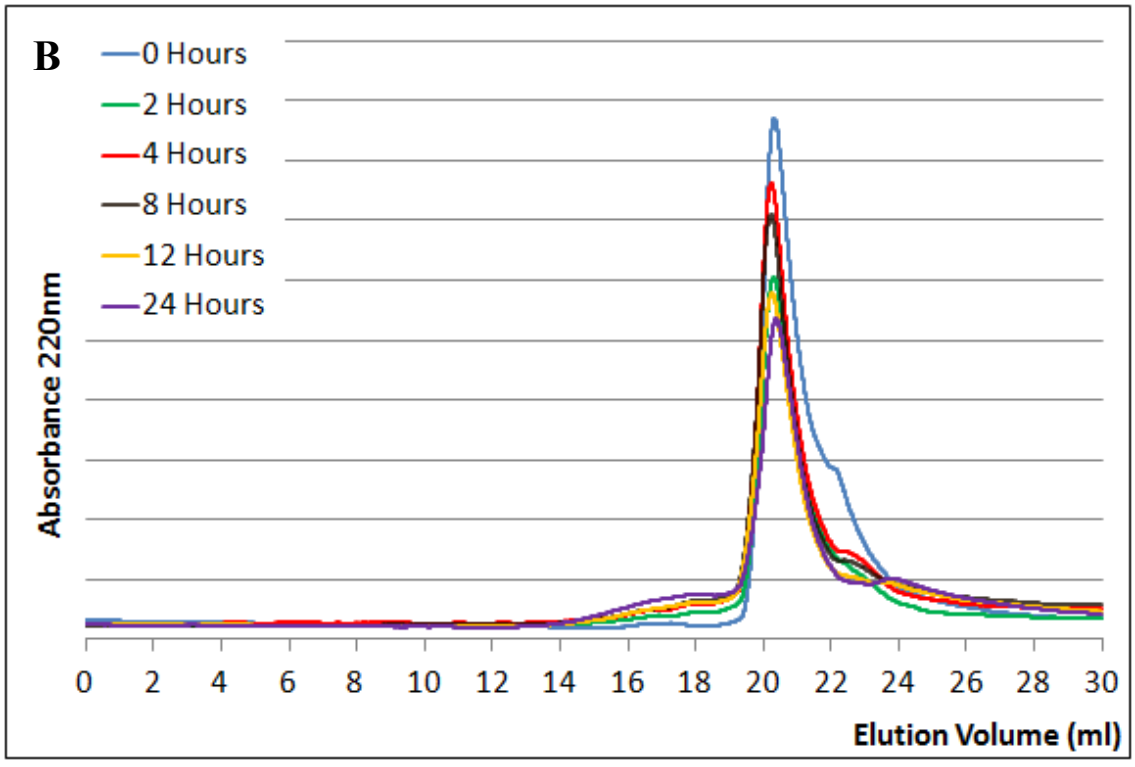
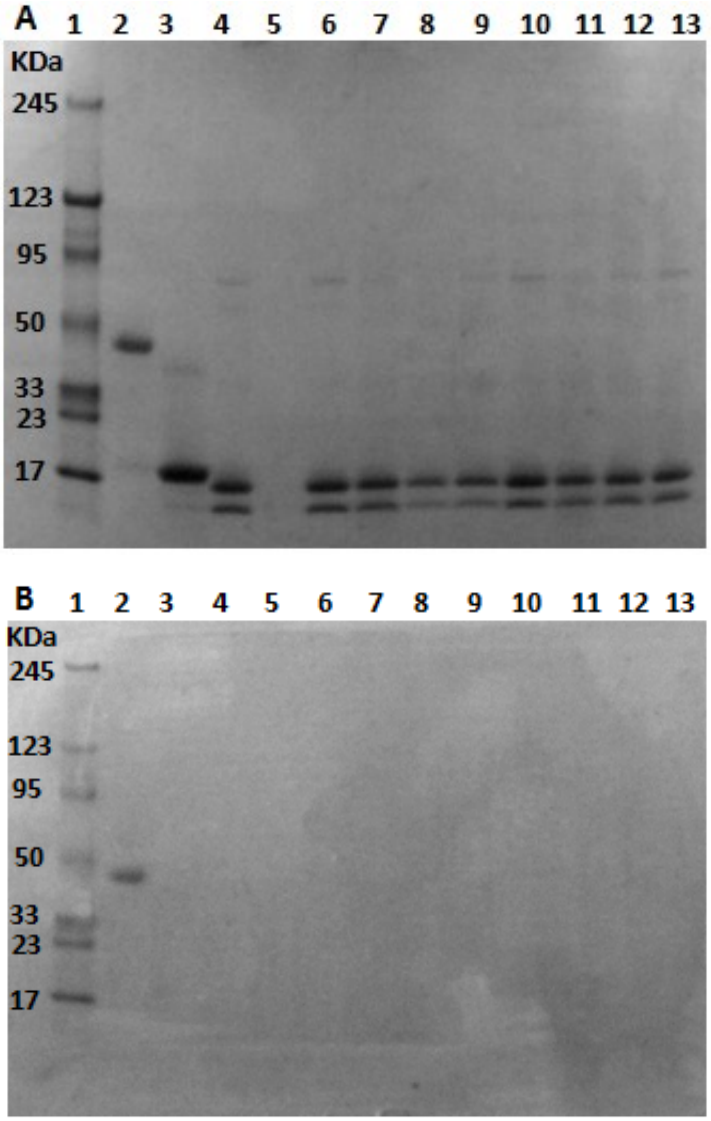



Figure 4. SDS-PAGE of unreacted and reacted WPI – Dextran solutions stained for protein (A) and glycoprotein (B). Lane 1: protein molecular weight standards; Lane 2: positive control for glycoprotein staining; Lane 3: negative control for glycoprotein staining; Lane 4: Whey Protein Isolate (raw material); Lane 5: 40KDa Dextran (raw material); Lane 6: 10mM Citrate, unreacted; Lane 7: 10mM Citrate, 24 hours; Lane 8: 50mM Citrate, 24 hours; Lane 9: 100mM Citrate, 24 hours; Lane 10: 10mM Phosphate, unreacted; Lane 11: 10mM Phosphate, 24 hours; Lane 12: 50mM Phosphate, 24 hours; Lane 13: 100mM Phosphate, 24 hours.



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## III-B. Production of Dextran – WPI nanofibers by needleless electrospinning

### Materials and Methods

Poly(ethyleneoxide) (PEO, Mw = 600KDa) and Dextran from *Leuconostoc* spp. (Mw = 100KDa) were purchased from Sigma Aldrich (Germany). Sodium dihydrogen phosphate dihydrate (>99% pure) and disodium hydrogen phosphate heptahydrate (>98% pure) were purchased from Carl Roth and Co. GMBH (Germany). Whey Protein Isolate (WPI, Lacprodan DI-9224) was a gift from Arla Foods Ingredients (Denmark).

PEO solutions were prepared by mixing PEO in distilled water at 40°C. The solutions were left for 4 hours on a magnetic stirring plate at 40°C until complete dissolution of the polymer.

Dextran – WPI solutions were prepared by mixing the appropriate amount of polymers into a 30mM Phosphate buffer at pH 6.5. Polymers were mixed into the liquid with a spatula until the solution was homogeneous; the solution was stored over night at room temperature overnight to obtain complete hydration of the macromolecules before electrospinning.

Microscopy images were obtained with a light microscope (Axio Scope.A1, Zeiss) equipped with a camera (Canon HAL100, AxioCam ICc3) and operated by AxioVision software (AxioVs40 V 4.8.2.0).

### Preliminary results and future perspectives

*Electrospinning setup evaluation*

The needleless electrospinning setup to be used in this study was first tested using PEO, a known spinnable polymer[1,2]. A solution of 6% PEO in distilled water was successfully spun at 46kV using a spinnerette – collector distance of 20cm. A light microscope image of the fibers obtained is shown in Figure 1. The diameter of the fibers obtained was around 0.5µm.

*Electrospinning of Dextran – WPI solutions*

The electrospinning of Dextran – WPI solutions was tested under different conditions of potential difference, spinnerette – collector distance

and solution properties such as total polymer concentration and ratio of Dextran to WPI. The polymer concentration in needleless electrospinning has to be balanced between reaching the critical polymer entanglement concentration (obtained with higher polymer concentrations)[3], and optimal solution viscosity, high enough for the solution to be picked up by the rotating spinnerette but low enough for fibers to be emitted from the solution surface. With an increase in solution viscosity, higher voltages are needed to obtain the spinning of fibers[4]. The ratio of Dextran to WPI is important for the conjugation step that follows fiber formation, for the production of conjugates, where a higher Dextran to WPI ratio was found to be better[5], but it is also important for the spinnability of the solution as Dextran is a neutral carbohydrate so increasing the concentration of WPI increases the electrical conductivity of the solution.

The first successful electrospinning of Dextran – WPI was obtained using a 2:1 Dextran – WPI ratio with an overall polymer concentration of 0.85g/ml. The spinnerette – collector distance was 18cm and the potential difference was 60kV. Spinnerette rotation was set at 50rpm whereas collector speed was 100rpm. The resulting fibers were not enough to produce a fiber mat, as in the case of PEO, but they were collectable. A light microscope image of the Dextran – WPI fibers is shown in Figure 2. The diameter of the fibers obtained was around 1μm and approximately 0.5mg of fibers were produced in 10 minutes of electrospinning.

*Future perspectives*

The solution properties of Dextran – WPI solutions still need to be optimized to obtain denser fiber mats, i.e. the generation of more material over time, by modulating further polymer concentrations and Dextran – WPI ratio, as well as the operating parameters of the electrospinning unit.

The obtained fibers will then be reacted for the production of Dextran – WPI conjugates. This project proceeds beyond the completion date of this Thesis.

**Conclusions**

The preliminary work shown thus far proves the potential application of needleless electrospinning for the production of Dextran – WPI fibres. The larger scale production of such fibers, compared to needle electrospinning, will pose the basis for the study of an industrially attractive method to form protein – carbohydrate conjugates to be used as functional ingredients in the food industry.

**List of Figures**

Figure 1. Light microscopy image at 100x magnification of electrospun Poly(ethyleneoxide) fibers (6% solution in distilled water).

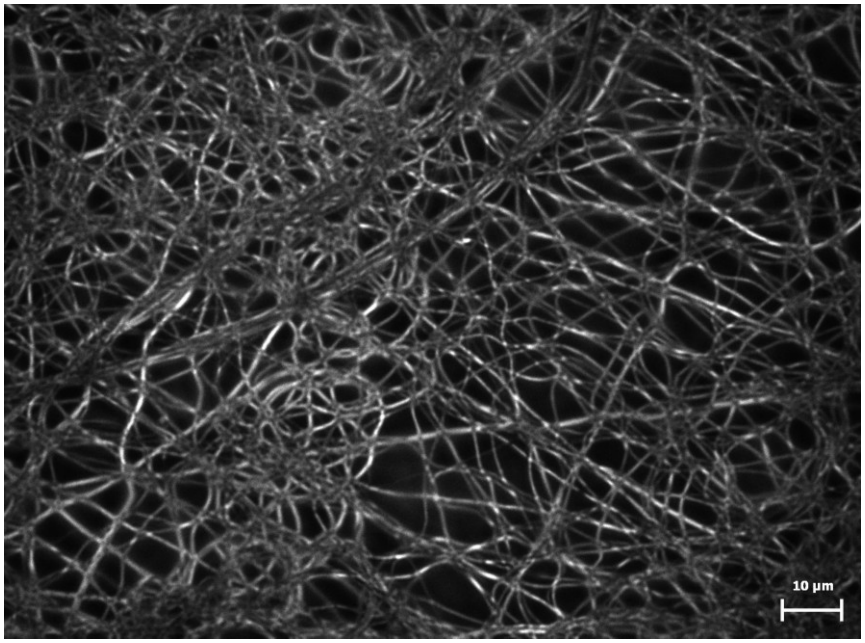
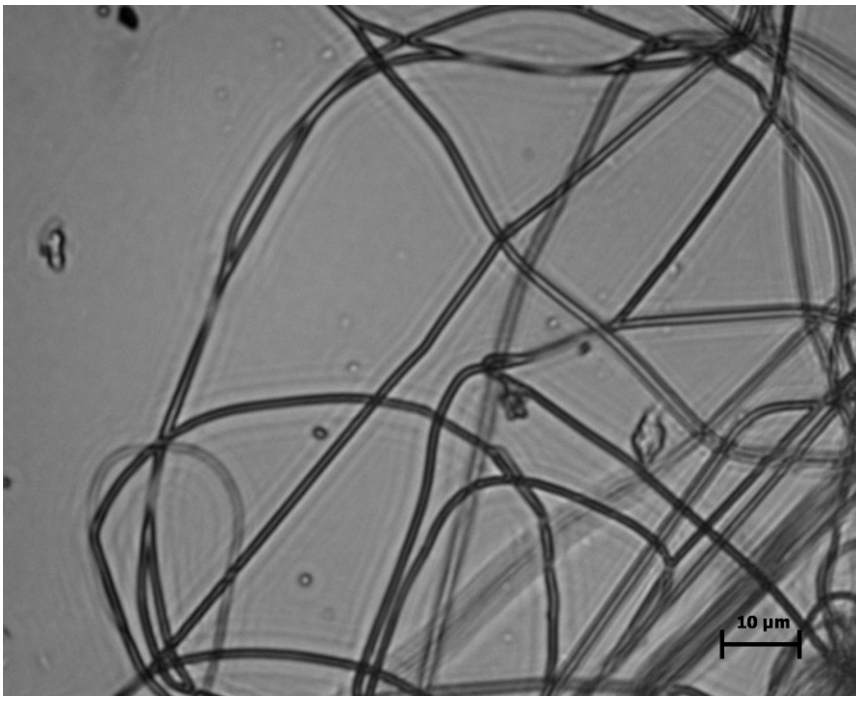



Figure 2. Light microscopy image at 100x magnification of electrospun Dextran – WPI fibers.



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