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## **PHYSICAL STATE OF SUGAR MATRICES AND AROMA-SUGARS INTERACTIONS AT NANO-SCALE**

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## ABSTRACT

Encapsulation at micro- or nano-scale is a new and versatile strategy to enhance the functionality of aroma and flavors by increasing their stability over usage and storage time and by controlling delivery and release. The achievement of a glassy state is critical for encapsulation of volatile compounds that become entrapped in the system during the proper vitrification process. Process variables, thermal history and compositional factors affect the retention ability and the physical properties of the low moisture/dry amorphous materials used for encapsulation. Design and development of flavor-encapsulated matrix require the definition of the technological functionality in the glassy matrix, the understanding of structure-function properties and of the interactions occurring at nano-scale that affect the retention of volatile compounds in the confined sugar matrices.

This research project is aimed to develop knowledge about the role of the physical state of disaccharides and the interactions at molecular scale on the release/retention of aroma compounds in low moisture-to-anhydrous glassy matrices. Experiments have been carried out on systems made of trehalose (TRE) as disaccharide and limonene (R-Limo) as model volatile compound.

The following aspects has been investigated: (i) the thermodynamic properties and interfacial properties of limonene in TRE aqueous solutions at different concentrations as preliminary investigation for the spray-drying experiments; (ii) the effects of different glass forming processes on the physical and structural properties of the glassy matrices and on the limonene retention. This part of the study has been carried out by applying spray-drying and milling/comilling processes. Glassy systems with different composition have been prepared from TRE solutions added with limonene or by processing crystalline TRE-b mixed with limonene in different ratios; (iii) the stability of the spray-dried and comilled glassy matrices upon storage under low moisture conditions.

Glassy TRE and TRE+R-Limo matrices differently prepared and formulated have been analyzed by instrumental techniques to determine the physical and structural properties and limonene retention. In particular, Differential Scanning Calorimetry

(DSC), Scanning Electron Microscopy (SEM), and Time-Domain NMR have been used, along with gas-chromatographic analysis (GC-MS) to quantify the limonene content. Glassy R-Limo microencapsulated TRE matrices have been obtained by spray drying and comilling with significantly different thermal, microstructural and mobility properties. Due to the different thermal history amorphous particles of different shape and size were obtained, with an effect of limonene on the size in the spray-dried particles. On the contrary, comilling determined the formation of a powder-like system made of particles of micron-size characterized by the presence of submicron, nano-droplets of limonene as in liquid-solid dispersion. DSC analysis evidenced a significant difference between the spray-dried and comilled samples containing limonene that could be partly ascribed to the different amorphization procedure and to the structural properties of the glassy low moisture products. Presence and content of limonene in the comilled amorphous systems did not determine changes in the  $T_g$  of the glassy liquid-solid dispersion, while it modified significantly the kinetics of cold-crystallization and melting at  $T > T_g$ .

NMR analysis highlighted a different molecular mobility and strength of interactions in the glassy matrices as a consequence of the process conditions and presence (and concentration) of the aroma.

Retention of limonene resulted significantly different in the differently prepared systems and higher in the comilled processed ones as a consequence of the different thermal history of the system during amorphization that favored the liquid-vapor transition of limonene during spray-drying. The enhanced surface properties and the increased tendency of limonene to escape in the vapor phase in trehalose solutions at increasing TRE concentration contribute to the higher processing loss.

From a structural and physical point of view results of this study seem to support the hypothesis that limonene, a relatively high hydrophobic compound, becomes encapsulated in the differently amorphised glassy, trehalose matrices as discrete sub-micron and nano droplets as a liquid-solid dispersion. The nano-dispersed limonene droplets are confined in the "free" volume of the matrix without modifying the main strength of the molecular interactions of the saccharide. In the comilled systems

limited amounts of limonene could also be partly present in the interspaces of the particles and/or adsorbed onto the surface of glassy and of the crystalline, not amorphised particles.

Loss of limonene occurred during storage could be partly determined by crystallization processes induced by residual water in the glassy samples, more relevant in the spray-dried samples. In the milled samples at short processing time (4 h), due to the not complete amorphisation of TRE, the limonene is dispersed in a system where amorphous and crystalline states of the disaccharide are simultaneously present and this could represent the main factor hindering the loss of the aroma during storage.

## RIASSUNTO

L'incapsulamento (micro-, nano-) è una moderna e versatile strategia tecnologica per migliorare la funzionalità di aromi e di altri composti attivi che permette di facilitare il loro impiego in sistemi complessi controllando la ritenzione ed il rilascio come pure di aumentare la loro stabilità nel tempo. Per conseguire l'effetto di incapsulamento, risulta critico l'ottenimento di matrici in stato vetroso poichè in questo stato fisico i composti volatili rimangono intrappolati ed immobilizzati all'interno della struttura disordinata amorfa ad alta viscosità. Le variabili di processo, la storia termica e la composizione dei materiali impiegati per l'incapsulamento ('carrier') influenzano la capacità di ritenzione dei composti attivi e le proprietà fisiche dei prodotti incapsulati finali che in genere presentano livelli di umidità molto bassi.

La progettazione e lo sviluppo di aromi incapsulati richiedono la definizione della funzionalità tecnologica dei composti volatili nella matrice vetrosa, la comprensione delle proprietà struttura-funzione e delle interazioni che si verificano a livello molecolare o di nano-strutture che possono influenzare la ritenzione dei composti volatili.

Questo progetto di ricerca ha l'obiettivo di sviluppare nuove conoscenze sul ruolo dello stato fisico e delle interazioni a livello molecolare che influenzano la ritenzione e il rilascio dei composti volatili odorosi in sistemi vetrosi a base di zuccheri semplici (disaccaridi) e aromi preparati con tecnologie di incapsulamento. Lo studio è stato condotto su sistemi costituiti trealosio (TRE) come disaccaride e limonene (in particolare, l'enantiomero R-) come composto volatile modello.

Sono stati studiati i seguenti aspetti: (i) le proprietà termodinamiche e le proprietà interfacciali di limonene in soluzioni acquose a diverse concentrazioni di TRE come indagine preliminare agli esperimenti di disidratazione per atomizzazione (spray-drying); (ii) gli effetti di vari processi di vetrificazione sulle proprietà fisiche e strutturali delle matrici vetrose contenenti limonene sulla loro stabilità e capacità di efficacia di incapsulamento e ritenzione dell'aroma. Questa parte dello studio è stata effettuata mediante l'applicazione di processi di essiccamento per atomizzazione e di macinazione (milling) e di co-macinazione (comilling). Sistemi vetrosi con diversa composizione sono stati preparati da soluzioni a base di TRE e limonene o a partire da TRE cristallino mescolato con limonene in diversi rapporti ponderali; (iii) la stabilità delle matrici vetrose ottenute per spray-drying e comilling durante la conservazione in condizioni di bassa umidità.

Le matrici amorfe a base di TRE e di TRE + R-Limo diversamente preparate e formulate sono state analizzate mediante varie tecniche strumentali per determinare le proprietà fisiche e strutturali e la ritenzione di limonene. In particolare sono state utilizzate la calorimetria a scansione differenziale (DSC), la microscopia elettronica a scansione (SEM) e la Time-Domain NMR; per quantificare il contenuto limonene sono state impiegate tecniche gascromatografiche (GC-MS).

I processi di spray-drying e di comilling hanno permesso di ottenere matrici vetrose a base di TRE con limonene incapsulato con significative differenti proprietà fisiche, termiche e microstrutturali. Nel caso dei prodotti ottenuti per essiccamento, per effetto della storia termica, sono stati ottenute particelle amorfe di forma e dimensioni differenti con un effetto sulle dimensioni delle particelle essiccate dato anche dalla presenza del limonene. Al contrario, attraverso il processo di comilling si è osservata la formazione di una matrice anidra in polvere a base di particelle di dimensioni del micron caratterizzate a loro volta dalla presenza di goccioline di limonene di dimensioni inferiori al micron come si può riscontrare in dispersioni liquida-solida. Le analisi DSC hanno evidenziato una differenza significativa tra i campioni ottenuti per spray-drying e comilling che può essere in parte attribuita alla diversa procedura di amorfizzazione e in parte alle proprietà strutturali delle matrici ottenute. La presenza ed il contenuto di limonene nei sistemi amorfi ottenute con la tecnologia del comilling non hanno determinato cambiamenti nel valore della temperatura di transizione vetrosa ( $T_g$ ) della materiale solido vetroso ottenuto, mentre hanno modificato significativamente la cinetica di cristallizzazione e di fusione osservati alle temperature al di sopra della  $T_g$ . Le analisi NMR hanno evidenziato una diversa mobilità molecolare e forza delle interazioni nelle matrici vetrose per effetto delle condizioni di processo e per la presenza (e concentrazione) dell'aroma nelle matrici amorfe. La ritenzione del limonene è risultata significativamente diversa nei sistemi disidratati o in quelli ottenuti per co-macinazione con una efficienza di incapsulamento maggiore in questi ultimi per un importante probabile effetto della diversa storia termica durante amorfizzazione che ha limitato le perdite del limonene a causa della transizione liquido-vapore di limonene che, al contrario ha luogo durante l'atomizzazione. Inoltre, il limonene ha mostrato interessanti proprietà tensioattive che risultano esaltate nelle soluzioni zuccherine possono aver influito sulla loro perdita durante il processo di essiccamento per atomizzazione. Considerando i risultati delle analisi termiche e microstrutturali questo studio sembra portare alla ipotesi che il limonene, composto volatile relativamente idrofobico, viene incapsulato nelle matrici vetrose a base di trealosio sottoforma

di goccioline di dimensioni sub-micron e di nano-goccioline a formare complessivamente una dispersione liquido-solido. Il limonene nano- e micro-disperso rimane confinato nel volume "libero" ("free volume") della matrice senza modificare le principali interazioni presenti a livello molecolare nel saccaride in stato amorfo. Nei sistemi ottenuti per comilling il limonene potrebbe anche essere in parte presente negli interspazi delle particelle e/o adsorbito sulla superficie delle particelle vetrose e dei cristalli eventualmente ancora presenti e non amorfizzati.

La perdita di limonene che è stata osservata durante la conservazione potrebbe essere determinata da processi di cristallizzazione favoriti dall'umidità residua nei campioni vetrosi, specie nel caso dei sistemi ottenuti per spray-drying. Nei campioni ottenuti per comilling con tempi di processo brevi (4 h), a causa della non completa amorfizzazione del TRE, il limonene si trova disperso in un sistema in cui il TRE si trova simultaneamente presente in stato amorfo e cristallino e questo potrebbe rappresentare il fattore principale che ha limitato significativamente le perdite dell'aroma durante la conservazione.

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## **PREFACE AND AIMS**

Flavor is one of the most important quality attributes that affects humans consumption and food acceptability. Most of the flavor properties of foods are due to odorous volatile organic compounds, their presence and concentration depending on raw materials, processes and storage conditions. Thermodynamic and kinetic aspects of the aroma release are function of the chemical, physical, physicochemical and structural properties of the food system as well as of the interactions occurring between the volatile and non-volatile compounds that, in turn may favor or unfavor the release and retention processes.

In food formulation flavor represents a main challenge and has become increasingly important in food product design (Uhleman and Reiss, 2010). Aroma compounds besides their volatility, that is necessary for their loss from the food system when kept in unconfined environments, present a relative high chemical reactivity and are unstable to heat and oxygen. The empirical development of specific aroma properties in food products to influence product properties has been achieved by simply mixing volatile compounds based on their chemical and sensory attributes generally in liquid form. This approach, however, has shown some limits in its application, lacking the knowledge of the physicochemical properties of the aroma compounds and of the food matrix, and the understanding of the interactions after the processing and/or diverse storage conditions.

Micro-encapsulation or nano-encapsulation is nowadays representing an interesting strategy to enhance the functionality of aroma and flavors by increasing their stability over usage and storage time as well as by controlling their delivery and release. The application of various conventional (freeze-drying, spray-drying) and novel (e.g. liposome encapsulation, spray drying, spray chilling) technologies in single or in combination allow to produce flavors encapsulated in the dry powder matrix characterized by enhanced functionalities (e.g. solubility) and shelf-life longer than the corresponding liquid state.

Encapsulation systems based on carbohydrates and, in particular, those at medium high molecular complexity (starch, maltodextrins) and hydrocolloids in a low-moisture, amorphous state, have showed to successfully be able to decrease the rate of both the release and the degradation over storage. By the proper choice of the carrier/encapsulant as single compound or in mix, controlled release from the food matrix upon processing or storage can be also achieved.

It is well acknowledged that the retention of aroma in carbohydrate encapsulated matrix is due to the formation of a low moisture, highly viscous, glassy matrix able to entrap the aroma compound in a finely disperse state, that could resemble a “solution”. Drying (desolvation) and physical/ mechanical actions able to determine the rapid quenching of the carrier are the main processes applied to this aim.

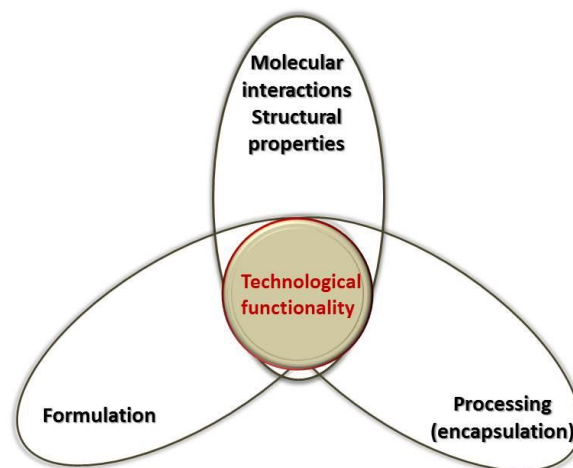
In the last decades the creation of new product characteristics by encapsulation is the main target for the flavor industry. This requires the application of new approaches in product design respect to those applied in the past that were based mainly on trial and error use of the existing technologies and materials. Design and development of a flavor encapsulated matrix requires the initial definition of its specific functionality in the formulated food system in which it will be added and included. This concept has been described by Ubbink and Kruger (2006) that suggested the so-called “retro-design” approach to the development of delivery systems of active ingredients in foods. The starting point of the design and development is the definition of the technological functionality and desired functionality, achieved by the choice of materials, technologies and their interactions over processing (Figure 1). This point requires the fundamental understanding of:

- the physical and chemical phenomena determining the stability, release and perception. In the last years, also the impact of digestive processes and of the *in vivo* effects the aroma compounds are becoming relevant.
- the specific chemical, physico-chemical and physical properties of the aroma compounds and of the carrier. This knowledge allow to better understand the potential interactions in the matrix between the various components and the

structural (nano-, micro-, macro-) properties of the encapsulated properties determined by the application of specific technological parameters.

- the role and effect of the parameters applied during process on the properties of the aroma and carrier constituents.

As a final remark, in the Ubbink and Kruger approach the functionality is defined solely on the analysis of the interactions of the components and is not related to a specific technology (Ubbink and Kruger, 2006).



**Figure 1:** Scheme of the technological functionality as due to the interactive effect of processing parameters, formulation and properties of the components in a matrix

In flavor encapsulation, starch and high molecular weight hydrolyzed derivatives (maltodextrins) are used due to their ability to easily develop low moisture/dry encapsulated aroma systems with interesting technological functionalities (Gharsallaoui et al., 2007). Low molecular weight saccharides (mono-, di-saccharides) are generally used as weighting agents in mix with other film- or glass- forming complex carbohydrates and other additives (surfactants). Small sugars (e.g., sucrose, glucose, maltose) and sugar alcohols (e.g., sorbitol, maltitol) are not used as sole encapsulating carrier for flavoring purposes even if several food products (e.g. candies,

confectionery) do exist where flavors are added. In these glassy systems aroma could be present in a dissolved, dispersed state depending on the nature of the aroma compound, the characteristics of the product and the applied process conditions.

The ability of low molecular weight saccharides in glassy state to retain aroma has been largely investigated in the early 70' and studies have been driven mainly by the need to give some insight to the "new" drying techniques under development (i.e. freeze-drying) and to define the main factor affecting the release and retention during desolvation processes at high temperature (spray-drying).

Two mechanisms are recognized to explain the retention of an aroma in these glassy materials: the "selective diffusion" and the formation during encapsulation and below a critical water content of "micro-regions" within an impermeable matrix where volatiles are entrapped. However, aroma compounds include molecules with different polarity and hydrophilicity; therefore, solubility, partition and phase separation of volatiles in the carbohydrate matrix have also to be taken into account.

Physical (e.g. viscosity, glassy or crystalline state, porosity) properties of the low moisture-dried carbohydrate-based matrices have been recognized as factors that could affect the liquid-vapor partition and the retention/release of aroma compounds in foods but these factors have been studied only to a limited extent and deserve specific investigations.

In recent times, a renewed interest to sugar-based glassy matrices as encapsulating materials has been observed in relation to the development of innovative sugar-based confectionery products with specific quality and sensory properties. Moreover, there is the need to better clarify the role of the physical state of the glassy sugar matrix and its changes due to storage time and to the presence of other co-solutes in the system.

The need to use saccharides different to the commonly used sucrose and/or starch hydrolysates (maltodextrins) for nutritional and health purposes (e.g. cariogenicity, high energy, etc.) has widen the interest towards alternative saccharides. Among them, trehalose has gained interest in recent time in the flavor sector, for its ability to

protect food matrices during various stressing processes (e.g. drying, spray-drying) and storage conditions (freezing), thereby with a reduction of aroma losses.

Trehalose (TRE) ( $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 1)- $\alpha$ -D-glucopyranoside) is a disaccharide that is accumulated by various microorganisms (e.g. yeasts) and multicellular organisms (e.g. tardigrades) as bioprotectant under extreme drought conditions (Cesàro, 2006; Kilburn et al., 2006). The formation of an intracellular carbohydrate glass, with high viscosity and hydrogen bond interactions stabilizes and protects the integrity of the biological complex structures and biomacromolecules (e.g. proteins). The bioprotective action of TRE is partly attributed to its high glass transition temperature of the amorphous phase, the highest among the disaccharides. Within the process conditions applied in food as well as other materials (pharmaceuticals, etc.) it could undergo to various phase transitions that are recognized as important for its bioprotective functionality. It has been demonstrated that in glassy state TRE has the ability to regulate its water content by local crystallization into dihydrate, therefore trapping water molecules. In general, when sufficiently plasticized by increases in temperature or water content, amorphous TRE may undergo re-crystallization to dehydrate or anhydrous forms, depending on temperature range and moisture content.

The chemical and physical properties and the corresponding functionalities in solid, glassy matrices could be of interest also in the development of new encapsulated flavor systems. However, very few is known about the physical properties of sugar-based glasses, and in particular those made with TRE, obtained in presence of aroma compounds as co-solute and the corresponding technological functionalities as well as the role of the process conditions applied to obtain the saccharide+aroma glassy matrices.

Specific investigations are also needed to achieve a clear understanding of the interactions occurring at nano-scale that could affect the retention of volatile compounds in the confined sugar matrices.

The general aim of this research project is to achieve an appropriate knowledge about the role of the physical state of disaccharides, and the interactions at molecular scale

on the release/retention of aroma compounds in systems at high moisture content (e.g. solutions) and in low moisture-to-anhydrous glassy states.

The project has been carried out in particular on systems made with trehalose as disaccharide and limonene as model volatile compound based on its physicochemical properties. For comparison purposes, in some experiments, other disaccharides like sucrose and lactose have been considered.

The study has been developed in three parts:

1. Thermodynamic properties and interfacial properties of limonene in TRE solutions at different concentrations. The aim of this part is to give some scientific background to the effect of TRE in affecting the release/retention of limonene in solutions and the amphiphilic properties of this apolar volatile compound. The results will contribute to the understanding of the retention of limonene in glassy systems obtained by desolvation (i.e. spray-drying, freeze-drying)
2. Study of the effects of different glass forming processes on the physical properties of the glassy matrices and on the retention of limonene. In particular the study was carried out by applying spray-drying and milling/comilling processes. The latter, while not largely used in the food area, has been showing interesting features to determine crystal-to-glass transformation and to develop interesting glassy materials with encapsulating potential. Glassy systems with different composition have been prepared by mixing TRE solutions or crystalline TRE-b and limonene in different ratios.
3. Study of the stability of the glassy matrices upon storage under low moisture conditions, evaluated in terms of aroma retention and physical properties changes.

Glassy matrices differently prepared and formulated have been investigated by instrumental techniques with the aim to characterize the physical and structural properties and the limonene retention. In particular, differential scanning calorimetry (DSC), Scanning Electron Microscopy (SEM), and Time-domain NMR have been used along with gas-chromatograph instrumentation to determine the limonene retention.

## **INTRODUCTION AND STATE OF THE ART**



## **I. LITERATURE REVIEW**

### **I.1 Aroma in foods: properties and technological functionality**

#### **I.1.1 Volatile aroma compounds and food quality**

Flavor is an important quality property that affects consumer's purchase and foods acceptance. Aroma, taste and mouthfeel perceptions have been recognized as components of the flavor properties of a food product being the former the most important one (Preininger, 2006). Aroma or odorants are volatile compounds released from the food systems in a sufficient amount to be perceived by the olfactory epithelium.

Foods are characterized by the presence of a number of volatile compounds that overall contribute to define the specific aroma pattern of the product along with its quality. From a qualitative point of view the presence and concentration of aroma compounds and the specific pattern of each food product depend on a wide range of factors including formulation and ingredient composition, processing conditions and storage. Changes in composition due to variations in the recipe and/or raw material quality characteristics along with process conditions (e.g. temperature and length in heat treatments like baking, pasteurization and sterilization) are highly affecting the overall aroma properties of a food product.

Moreover, during storage aroma pattern could be modified depending on various phenomena and processes including loss due to their spontaneous tendency to partly in the vapor phase; chemical degradations (oxidation, hydrolysis, reactions with other volatile and non-volatile compounds); generation of new volatile odorous compounds due to degradative reactions and processes of microbial, enzymatic and chemical origin that in the majority of the cases impair the overall quality of the food and its acceptance.

Thus, main efforts are made by food manufacturers to both improve the aroma properties of the food products as well as to maintain them over processing and storage conditions. In formulated food products this could be obtained by the choice

of high quality raw materials and ingredients with high odorous potential and aroma content as well as the addition of aroma compounds whose use is regulated by law (EC Regulation N<sup>o</sup> 1334/2008). In this field a large availability of aroma-based materials are nowadays available, either as liquid solutions or dispersions or as powder-like, low moisture products obtained by various encapsulation techniques. Increasing is the interest towards the encapsulated aroma products as they better preserve the sensory properties over storage time and/or processing. Moreover, encapsulated aromas depending on their nature and type, could also contribute to improve the overall sensory properties of the food products thanks to the possibility to modulate the release/retention of the aroma compounds depending on the quality properties of the food in which they are added and the expected functionality.

### **I.1.2 Thermodynamic and kinetic aspects of aroma release/retention**

Kinetic and thermodynamic mechanisms control the rate of release and the concentration of volatiles in the vapor phase in equilibrium with a food (Pittia et al., 2015; Grab and Gfeller, 2000). The static equilibrium distribution of flavor compounds occurs between the different phases of a product comprising the solid food matrix (e.g. starch, cellulose, protein), the hydrophilic liquid phase (water, solutions) or lipophilic liquid phase (oil) and the gas phase (air). The distribution is controlled by the phase partition coefficients of the volatile compounds that affect their volatility, presence and concentration in the gas phase (van Ruth et al., 2000).

The dynamic, kinetic component of the release is influenced by physical (viscosity, rheology, texture) and structural properties (e.g. porosity, interfaces) of the food matrix and controlled by the rate of diffusion (mass transfer) of the aroma molecules through the matrix and its interfaces.

Chemical nature of the aroma compounds, composition, and structure of foods are main characteristics that influence the transfer of aroma compounds within the foods and their release (Seuvre et al. 2006; Cayot et al. 2008).

Besides the intrinsic characteristics of aroma and food matrix and the environmental factors, interactions occurring between volatile and non-volatile compounds (in

particular, food macromolecules like proteins, hydrocolloids, and carbohydrates) are major factors that limit the release of volatiles, and thus their perception. This is demonstrated by a number of studies in this field reported in the literature papers, reviews and topic-specific book chapters (e.g. Naknean and Meenune, 2010; van Ruth and Roozen, 2009; Preininger, 2006; Delarue and Giampaoli, 2006).

Different are the binding processes and of different energy occurring between non-volatile and volatile molecules that include (Le Than et al., 1992):

- Covalent bonds (high energy, 100 kcal/mol, stable)
- Hydrogen bonds (moderate, 2-4 kcal/mol, polar)
- Hydrophobic bonds (low energy, solvent entropy-driven, apolar)
- Inclusion complexes (variable energy, complex contributions).

Their occurrence and relative importance in affecting the kinetics and thermodynamics of the release of a volatile compound in the vapor phase depend on many factors and in particular on the nature of the ligands and of the substrate as well as the environmental conditions.

- **Thermodynamic and kinetic aspects of aroma release/retention in high moisture and solutions of carbohydrates**

In carbohydrates, interactions with aroma compounds are in general characterized by weak energy, depending on various factors such as the nature and concentration of both aroma and carbohydrates that affect both the kinetics and the concentration in the vapor phase in equilibrium above the matrix.

While complex polymeric saccharides (e.g. starch) have been widely investigated for their role in affecting the release/retention of volatiles in food matrices, scarce attention has been given to that of small saccharides (i.e. mono- and disaccharides).

Carbohydrate polymers include a wide range of molecules (starches, pectins, gums, modified cellulose and hydrocolloids) that may differently influence the release and retention of aroma compounds by affecting both thermodynamic mechanisms and kinetics with effects that may depend on their concentration. By acting as thickening

agents they change the viscosity of the aqueous phase and reduce the mass transfer kinetics of the vapour release in the gas phase.

Starch, after gelatinization of the native granules, has been seen able to entrap volatile compounds physically and reversibly (Escher et al., 2000) as the amylose interacts with apolar ligand molecules by the formation of inclusion complexes (Heinemann et al., 2001, Rutschmann and Solms, 1990). In these structures the volatile molecules with linear moiety are embedded in the hydrophobic cavity of the amylose helix whereas bulky ligands may be located between the amylose helices (Helbert and Chanzy, 1994). When more volatile apolar molecules may be present in the dispersion, competitive phenomena for the hydrophobic cavity may occur.

Various studies have highlighted the role of maltodextrins in affecting the retention/release of volatiles with effects that depend on both molecular weight and nature of the aroma. Maltodextrins at low Dextrin Equivalent (DE) present a higher ability of reduce the vapor partition of an aroma due to specific interactions between the volatile and the non-volatile molecules and/or entrapment phenomena (Naknean and Meenune, 2010; Bangs and Reineccius, 1981).

As regards small saccharides (mono-, di-saccharides) it has been generally recognized that they exhibit a salting-out effect that causes an increase in volatility of the volatile compound relative to that in water (Voilley et al., 1977). Sugars, and in particular sucrose, have shown to affect the partition of volatile compounds especially of the more polar ones, due the reduction of the water freedom of the condensed liquid phase leading to the so called "salting out" effect (Draux and Voille, 1997).

However, several studies on the volatility of aroma compounds in sucrose solutions appear to be in conflict to the aforementioned behavior showing both depression and enhancement of volatility (Nahon et al., 2000; Hanson et al., 2001, Robert et al., 1996). Voilley and Bosset (1986) determined a higher partition coefficient of the polar volatile compounds diacetyl, acetone, ethylacetate and 2-propanol in 50% glucose solutions at 25°C. A trend on the increased release due to the polarity has been observed as the more polar aroma compounds showed an increased concentration in the vapour phase due to the change of the water freedom in the solution affecting the vapor partition of

the volatile compound. On the contrary, the less polar ones showed a retention. Furthermore, an increase in threshold perception of non-polar compounds was observed in limonene with added sucrose (Ahmed et al., 1978) and a decreased volatility of the same volatile in systems added with glucose and sucrose (Massadi and King, 1973).

Nahon et al. (2000) studied the effect of sucrose on the mass transfer and partition of volatiles of different polarity in sucrose solutions at concentration up to 60% at 37°C and they did not find a significant effect of sucrose and its concentration on the partition coefficient of ethyl hexanoate while it was observed for octanal, a volatile compound more apolar than the former one, with a decrease of the gas/solution partition coefficient at 37°C at increasing sucrose concentration up to 60 %w/v. An increase of the release of some apolar compounds present as flavor mix intended for soft drink including linalool and menthone was observed in solutions of inverted sugar and sucrose especially at concentrations higher than 40 % while limonene was not affected by the same sugars up to 60% (Hansson et al., 2001).

However, subsequent studies have highlighted the role and relative importance of other factors that could affect the release of volatiles in small saccharides-based aqueous systems. The solubilisation of a sugar moiety in water is associated with the formation of hydration shells of water surrounding the solute molecules (Starzak et al., 2001). Depending on the solute type and concentration and related water/solute interactions related strength, the ratio between free (non-associated) and bound (associated) water should decrease with increasing solute concentration. If no adsorption occurs between the aroma compound and the hydrated sugar, the vapour partition of the former will be simply affected by its concentration in the “free water” fraction.

Depending on the water affinity of the specific sugar and the specific chemical and physico-chemical properties of the volatile compound, thus, a different effect on the liquid-vapor partition could be expected. On the other hand a retention effect due to the addition of sugars in the liquid phase could be attributed to changes other than

those related to the water state due to the chemical and physico-chemical properties of the sugars especially when complex aqueous system are taken into account.

Pittia et al., (2012) evidenced that different saccharides (sucrose, lactose, glucose) added to ready-to-drink coffee drinks present a different ability to affect the retention and release of the volatile compounds of the differently sweetened beverages with differences depending on both the sugar type and the aroma chemical properties, and, in particular, their hydrophilicity. Modifications of the physicochemical properties of the aqueous phase due to the saccharides may induce changes in the interactions between the volatile and the non-volatile induced by these solutes in the aqueous phase that, in turn, affect the vapor partition and thus their perception (Delarue and Giampaoli, 2006; Adhikari et al., 2006). The amphiphilic character of sugar implies that in aqueous solutions, besides the salting-out effect due to the presence of hydrophilic regions of the saccharide moiety, a “salting in” effect due to interactions of the aroma compounds, especially those more apolar, in the less polar regions could occur.

Polar water-soluble molecules can moreover enhance the solubility of the more hydrophobic molecules by changing the structure around the non-polar substance by changing the water solvency and/or by creating intramolecular non-polar environment for hydrophobic molecules (solute interactions) (Yalkowsky and Banerjee, 1992). The co-solutes increase or decrease the solubility depending on their concentration and the polarity of the volatile compound and the molecular configuration of the cosolute and volatile (Covarrubias-Cervantes et al., 2005; Jouquand et al., 2004). Covarrubias–Cervantes et al. (2005), evidenced a change of the solubility and the liquid-vapour partition of some volatile compounds when added in solutions containing sugars and polyols like sucrose, glucose and sorbitol as a function of their concentration.. Glucose and sucrose in aqueous solutions have been reported to change the Henry’s constant values by Copolovici and Niinemets (2007), who showed a salting-in effect by sucrose and glucose (1 M) and a salting-out effect by sorbitol when the limonene and linalool were considered.

- **Thermodynamic and kinetic aspects of aroma release/retention in low moisture/dried/anhydrous carbohydrate systems**

Low water content carbohydrate matrices in glassy state are considered valuable in the flavour sector due to their ability to retain volatile aroma compounds. This has been largely exploited in the flavor encapsulation sector by the development of amorphous carbohydrate mixtures such as sucrose and maltodextrins with water content < 5% as encapsulating matrices (Gunning et al., 2000).

Encapsulation effect is exerted by carbohydrates exerting “impermeable” properties toward the volatile aroma compounds. Moreover, aroma compounds must be not too soluble in the matrix to avoid that the particles become sticky and liable to caking, a behavior associated with the surface tension and viscosity of the carbohydrate matrix above the glass transition (Wallack and King, 1988; Slade and Levine, 1995; Gunning et al., 2000). The properties of the glassy state in food systems has been subject of increasing attention in food industry for the development, formulation of flavor-based products. In particular, the stability of glassy state has been widely reviewed in the literature (Roos, 2010; Le Meste et al., 2002).

Studies on the retention of volatile, aroma compounds in freeze-dried powders obtained from simple saccharides solutions (in particular sucrose, glucose) have been carried out in the earlier 70's, mainly to deepen the knowledge and performances of the drying and freeze-drying processes. Transformations of solution structures in freeze-concentrated systems were also found to contribute to flavor retention affecting collapse in freeze drying (Bellows & King 1973; To & Flink 1978; Levine & Slade 1989). The formation of non-crystalline glassy structures in sugar dehydration has been well documented (Roos & Karel 1991a; Roos 1995).

Three main theories were proposed about the mechanisms by which volatiles are retained during drying and storage by the formation of low moisture, glassy matrices:

- the sorption of the aroma compound on specific matrix sites (Rey and Bastien, 1962)
- diffusion-based mechanisms (Thijssen and Rulkens, 1969, King and Chandrasekaran, 1971). This theory is based on the different diffusion coefficients of

water and volatiles and their dependence upon moisture content in such a way that the diffusion of the volatile is much smaller than that of water when the water content is low.

- Entrapment mechanism in “micro-regions” (Flink and Karel, 1970). The authors postulated that during freeze-drying the formation of “microregions” containing high concentrations of the carbohydrate and of the volatile compounds occur (Flink and Gejl-Hansen, 1972). Upon moisture decrease by freeze-drying (FD) a molecular association of the carbohydrate occurs via hydrogen bonding. This determines the formation of a complex structure made of a carbohydrate-rich phase containing a dispersed volatile-rich phase, that controls the permeability to water and to organic volatile molecules with an efficiency that depends on the local moisture content and structural properties (Flink and Karel, 1972). Under this hypothesis, n-alcohols ranging from methanol to pentanol were differently retained in low moisture carbohydrates (glucose, maltose, sucrose or lactose) as a function of the sugar type, specific physico-chemical properties of the aroma compound and process conditions. In particular, a higher retention was observed in sucrose FD powders for tert-butanol that, among the volatile considered, was one of the most hydrophobic (higher logP) with low vapor pressure (Flink and Karel, 1972).

These theories were further developed and improved by the studies of Gunning et al. (1999) and Gunning et al. (2000), where factors like the dispersion state, the affinity of the volatile compound and the carbohydrate as well as the solubility of the aroma compound in the concentrated sugars solutions were also taken into account. These studies evidenced the relevant role of the partition of the volatile compound in the carbohydrate-rich phase (specific affinity) and of the water content even in systems in amorphous state as it could affect the solubility of the volatile in both phases.

Nowadays, the glass transition “concept” introduced in food science and technology by Slade and Levine in the 80’s (Levine and Slade, 1986) is giving some more insight to the retention mechanisms of aroma volatile compounds in low moisture systems whose entity depends on the physical state of the carrier/encapsulant/matrix (Goubet et al., 1998). Low moisture glassy and crystalline states differently influence the ability to



limit matrix-to-air release and the corresponding efficiency of retention. Rapid or quenched dehydration (spray-drying, freeze-drying) allows to obtain matrices in glassy state (Whorton and Reineccius, 1995, Roos and Karel, 1991), characterized by a very low mobility of the carrier molecules. Under these conditions, release of volatile encapsulated/entrapped components occurs primarily via Fickian-like diffusion through the pores in the matrix and, from the matrix, through the exposed surface. The amount of the volatile released depends on the composition of the matrix, its nano- and micro-structure properties (pore size, particle size, thickness and area around the entrapped volatiles). Thus, release or retention of volatile compounds in a glassy state matrix depends upon the rate at which aroma can migrate to the surface (diffusion) more than upon the relative volatility of the aroma (Whorton, 1995).

Above the glass transition temperature of the matrix, achieved either by heating or by increasing the content of a plasticizer (water, in general), a transition from the solid glassy to a liquid-like rubbery state occurs. Softening of the material is usually ascribed to a decrease in the cooperativity of the hydrogen bond networks responsible for the main structural forces in the amorphous products (Roos, 2010; To and Flink, 1978; Whorton C, Reineccius GA. 1995; Goubet et al., 1998). Upon increasing water uptake, several phenomena, like swelling, agglomeration, caking and dissolution, could occur eventually causing the destruction of the microregions immobilising the volatiles and the release of the diffusing aroma (Chirife and Karel, 1974; Rifai and Voilley, 1991).

During moisture uptake and/or temperature increase the viscosity of the plasticized system entering in the rubbery state collapses. Under these conditions, the porosity of the rigid matrix could be lost and the diffusion through the matrix could be even reduced (Goubet et al., 1998). In some cases however, collapse could determine a re-encapsulation of the aroma compounds (Labrousse et al., 1992, Whorton and Reineccius, 1995) and in this state volatile release occurs, again, under Fickian-like conditions.

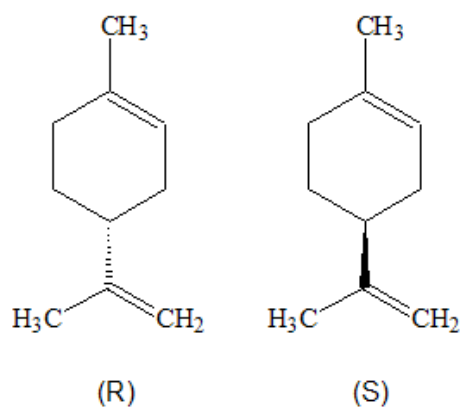
In crystal forming systems, the increased mobility of the system after collapse may lead to crystallization with kinetics described by the William-Landel-Ferry equation that lead, by cross-linking mechanisms, to highly ordered and dense matrices. Volatiles

are excluded from the crystallized matrix to the surface and the retention of the aroma is decreased (Labrousse et al., 1992; Levi 1995).

In crystalline systems, volatiles could be retained via adsorption mechanisms and the entity depends on the nature of the specific solid and aroma compound. By applying modern dry-milling techniques (e.g. co-grounding and solid state activation) it has been possible to modify, by increasing, the volatile adsorption ability of solid matrices by keeping its crystalline state (Calabretti et al., 2003).

### I.1.3. Limonene

Limonene occurs naturally in certain trees and bushes and, along with others monoterpenes, is released in large amounts mainly to the atmosphere, from both biogenic and anthropogenic sources. Limonene is also known as the main odor constituent of citrus fruits and its name originates from the lemon. The rind of lemon, as that of other citrus fruits, contains considerable amounts of this compound, which contributes to their characteristic flavor. Limonene is present also in dill, caraway, fennel, and celery and in turpentine.



**Figure I.1.** *R-(+)-limonene and S-(-)-limonene*

Limonene is a colorless liquid at room temperature. It is a hydrocarbon, classified as a cyclic terpene, and exists as two optical isomers, R-(+) and S-(-)-limonene, and the racemic mixture dipentene. The purity of commercial R-limonene is about 90–98%.

The structural formula for limonene is given in **Figure I.1**. R- and S-limonene enantiomers should differ only in those properties that rely on molecular dissimetry, while other physico-chemical properties coincide (**Table I.1**). However, commercial products are often given with some different chemical and physico-chemical properties because of different purity and contaminants.

Since sensory properties are consequence of molecular binding with biomacromolecular receptors, the perceived flavor of the two enantiomers is different. The more common (R)-isomer presents a fresh citrus, orange-like odour while its (S)-enantiomer has a harsh, pine or turpentine-like one. They are characterized by different odor threshold equal to 200 ppb and 500 ppb, respectively.

Limonene due to its chemical and pleasant sensory properties find applications in various manufacturing sectors and added as flavor and fragrance additive in cosmetics, pharmaceuticals, perfumes, household cleaning products. It is also used as a solvent in degreasing metals prior to industrial painting, for cleaning in the electronic and printing industries, and in paint as a solvent. Limonene is increasingly being used as a “green” solvent as it is biodegradable and it is also used as botanical insecticide. As it is combustible, limonene has also been considered as a biofuel.

As main flavoring constituent of citrus, R-limonene is largely used in food manufacturing and, in particular, added both as chemical and citrus oil, as component of aromas to be added in formulated products thereby contributing to a citrus-orange flavor note.

(R)-Limonene or its metabolites could cause skin irritation and have a sensitizing potential following its widespread use in various consumer products. However, based upon conventional determination of lethal dose ( $LD_{50}$ ) and repeated-dose toxicity studies when administered orally to animals, it is designated as a chemical with low adverse effects; is classified by the U.S. Food and Drug Administration (FDA) as Generally Recognized as Safe (GRAS) (Kim et al., 2013).

For humans food is the principal source of exposure to limonene and, on average, the ingestion of limonene was calculated to be 0.1 mg/kg body weight per day. Based on available data and within these consumption values, the exposure to this chemical

does not represent a significant risk to human health. Noticeably, limonene has also been studied for its anti-carcinogenic properties (WHO, 1998).

Limonene does not have functional groups for hydrolysis and its cyclohexene ring and ethylene group are known to be resistant to hydrolysis. On the contrary, it may degrade as it is prone to oxidation via radical reactions. The oxidized derivatives present sensory properties different than the precursor compound and thus impair the sensory properties of the food in which the aroma is present.

Physical and chemical data on limonene presented in **Table I.1** were taken from the Concise International Chemical assessment document edit by the WHO (1998).

The vapour pressure of limonene is rather high and its solubility in water is low, giving a high value of the Henry's law constant, which predicts a high rate of vaporization of limonene.

	<b>R-limonene</b>	<b>S-limonene</b>	<b>Dipentene</b>
<b>CAS no.</b>	5989-27-5	5989-54-8	138-86-3
<b>Chemical name</b>	(R)-1-methyl-4-(1-methylethenyl) cyclohexene	(S)-1-methyl-4-(1-methylethenyl) cyclohexene	1- methyl-4-(1-methylethenyl) cyclohexene
<b>Empirical formula</b>	C <sub>10</sub> H <sub>16</sub>	C <sub>10</sub> H <sub>16</sub>	C <sub>10</sub> H <sub>16</sub>
<b>Molecular weight</b>	136.23	136.23	136.23
<b>Melting point (°C)</b>	-74.35	-74.35	-95.9
<b>Boiling point (°C)</b>	175.5-176	175.5-176	175.5-176
<b>Density (g/cm<sup>3</sup>, 20°C)</b>	0.8411	0.8422	0.8402
<b>Vapour pressure (Pa at 20°C)</b>	190	190	190
<b>Water solubility (mg/litre at 25°C)</b>	13.8 <sup>b</sup>	-	-
<b>Henry's law constant (kPa m<sup>3</sup> /mol at 25°C)</b>	34.8 <sup>c</sup>	-	-
<b>Log K<sub>dw</sub></b>	4.23 <sup>d</sup>	-	4.83 <sup>e</sup> (limonene)

**Table I.1:** Chemical and physicochemical properties of limonene (WHO, 1998).

## **I.2 Encapsulation and stability of encapsulated glassy matrices**

### **I.2.1. Role of encapsulation in food processing**

Encapsulation is defined as a process in which tiny particles or droplets are surrounded by a coating or embedded in a homogeneous or heterogeneous matrix, to give small capsules with many useful properties (Pittia and Gharsallaoui, 2015). Microencapsulated ingredients are totally enveloped in a coating material thereby conferring useful or eliminating useless properties to or from the original ingredient (Gharsallaoui, 2007).

By encapsulation sensitive ingredients or “core” materials and ingredients could be physically enveloped in a protective matrix or “wall” material that allow their protection against degradative reactions, volatile loss, or nutritional deterioration. Besides its primary role on stabilization and protection, microcapsules could play a main role in the controlled release of their contents by delaying the rate or by maintaining constant the release over prolonged periods of time (delayed and sustained release) (Lakkis, 2007, Sanguansri and Augustin, 2010). For these reasons, one of challenges of the microencapsulation is to preserve stability of the encapsulated ingredients during processing and storage and to release these ingredients at a given physicochemical conditions.

In the food field microencapsulation is a technique by which liquid droplets, solid particles or gas compounds are entrapped into thin films of a food grade microencapsulating agent. The core may be composed of just one or several ingredients and the wall may be single or multi-layered. The retention of these cores is governed by their chemical functionality, solubility, polarity, and volatility (Gharsallaoui et al., 2007).

Microencapsulation in food industry is applied for a series of aims including: reducing the core reactivity with environmental factors; decreasing the transfer rate of the core material to the outside environment; promoting easier handling; controlling the release of the core material; masking the core taste; diluting the core material when it should be used in only very small amounts; separate components within a

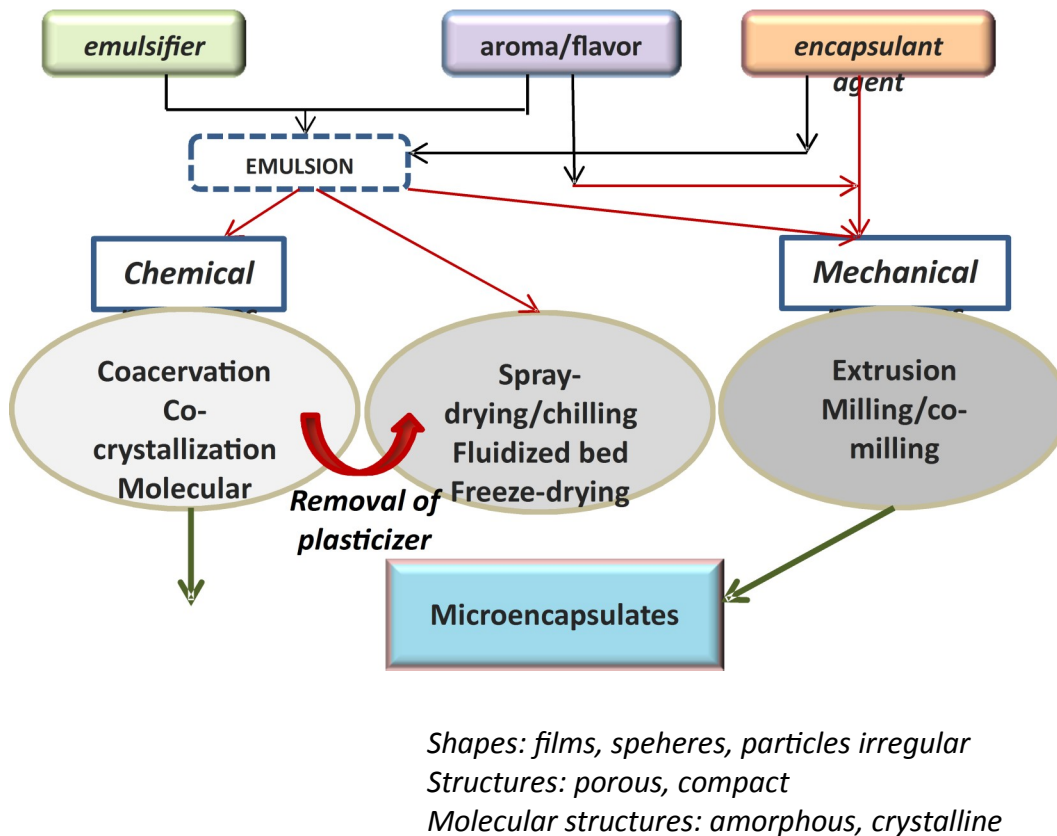
mixture that will otherwise reach with one another (Ray et al., 2016; Sanguansri and Augustin, 2010, van Ruth and Roozen, 2010).

Microcapsules are in general small spheres with diameters comprised between a few micrometers and a few millimeters. However, materials and technologies used are affecting shape and size of the formed microparticles. Depending on the physico-chemical properties of the core, the wall composition, and the used microencapsulation technique, different types of particles can be obtained: simple sphere surrounded by a coating of uniform thickness; particle containing an irregular shape core; several core particles embedded in a continuous matrix of wall material; several distinct cores within the same capsule, and multi-walled microcapsules (Gibbs et al., 1999).

Mechanical/physical and chemical processes could be applied to produce a range of microencapsulated food ingredients. Among the formers, emulsification, spray-drying, spray-cooling, spray-chilling, freeze-drying, fluidized-bed coating, centrifugal extrusion, spinning disk, pressure and hot melting extrusion co-crystallization, are included. Chemical-based technologies comprise ionotropic gelation, simple or complex coacervation, rotational suspension separation, liposome entrapment, interfacial polymerization, molecular inclusion, among others.

Encapsulation represents an important tool to protect volatile and sensitive and reactive food components like aroma and flavor compounds as well as to control their release at the right time and the right place in a standardized, functional form (van Ruth and Roozen, 2010; Andrade et al., 2008; Porzio, 2004). Additional benefits of encapsulating flavors include also the conversion of a liquid flavor into an easily dispensable and easy to store powder (Porzio, 2004).

Depending on the technologies available, different are the procedures required to allow flavor encapsulation as well as the initial system properties (high moisture/solution/dispersion versus low moisture/anhydrous) and compositional complexity as summarized in **Figure I.2**.



**Figure 1.2.** Processes and techniques to obtain encapsulated matrices

### 1.2.2. Encapsulation technologies by moisture removal (drying)

Encapsulated matrices could be obtained from high moisture solutions/dispersions by removal of the excess of water to obtain low moisture products, which are characterized by higher stability over storage time than corresponding initial systems. Among those available, spray drying and freeze-drying are currently the most used. Spraying methods are the microencapsulation techniques most commonly applied in the food industry and are employed to encapsulate a wide range of hydrophilic and lipophilic food ingredients. In the flavor sector spray-drying remains the dominant process technology of microencapsulation. On the contrary, freeze-drying, generally used for dried, high quality and value food matrices is used only to a limited extent for flavor encapsulation purposes (Yang et al., 2011).

Microencapsulation of flavors by spray drying implies the transformation of a feed from a liquid state (solution, dispersion, emulsion) to a low moisture, particulate form. Generally the process comprises three main steps: (i) preparation of the dispersion or emulsion to be processed, (ii) homogenization of the dispersion and (iii) atomization of the mass into the drying chamber.

Various are the factors that play a role in affecting the retention of the volatile compounds in the dried powders and main care has to be taken to avoid flavor loss during drying because high temperatures are often used during processing (Liu et al., 2001). The characteristics of the initial emulsion (interfacial composition, droplet size) and its stability, composition and concentration of the carrier that will form after drying the wall material or dispersant matrix are critical aspects related to the feeding system (Liu et al., 2001, Sosa et al., 2014). Encapsulating/carrier materials are generally selected based on the properties of the component to be encapsulated and the more largely used are proteins and carbohydrates, alone and/or in combination. On the other side, process conditions like atomization temperature and equipment features could contribute in favoring flavor loss during drying.

From a physical point of view spray-drying processes determine the formation of glassy structures from atomized liquids and rapid dehydration of the fine particles (Bhandari & Howes 1999, Roos 2002). Proper process conditions are required to allow solidification of particle surfaces at the initial conditions in the drying tower to retain free-flowing properties of particles toward powder outlet and to avoid stickiness and caking of particles in the equipment (Roos, 2010).

The formation of solid structures implies a rapidly increasing viscosity at the particle surfaces to extend contact times required for stickiness and adhesion (Downton et al. 1982). This can be achieved by glass-forming solutes such as lactose or cosolutes like maltodextrins or proteins (Shi et al., 2015; Roos 1995). By spray-drying the glass-forming solutes become a solid continuous phase that can entrap volatiles and flavors as well as encapsulate dispersed components (Zhou and Roos, 2012; Vega & Roos 2006, Gharsallaoui et al. 2007).



Process conditions applied to encapsulate flavor compounds must be generally optimized in order to achieve a high retention of the volatiles on the dried products. The proper choice of the encapsulant/carrier agent along with the colloidal properties of the dispersed lipophilic phase in the initial high moisture system are thus a critical aspect to limit aroma loss during processing.

Freeze-drying is the processing technique for dehydration of thermolabile compounds and used only to a limited extent for encapsulation and formation of glassy, low moisture particles due to the high energy input, long processing time and high costs compared to other drying methods (Ray et al. 2016). However, by freeze-drying, starting from a high moisture system in frozen state, high aroma/flavor retention could be obtained thanks to the different kinetics of the water removal from the matrix during drying under the process conditions at low temperature and pressure. Freeze-drying has been extensively studied in the 70's in order to deepen and clarify the mechanisms that allow the retention of volatile, aroma compounds in the corresponding dried, glassy matrices (Chandrasekaran and King, 1972, Flink and Karel, 1970, Flink and Karel, 1972).

### **I.2.3. Encapsulation from low-moisture/anhydrous systems**

In the food area few techniques are available to determine encapsulation of components of interest starting from low moisture-to-anhydrous matrices; generally they are based on mechanical and physical actions and use carbohydrates as carrier materials. In this framework melt injection and melt extrusion are among the technologies mostly used. The mix of the active compound and carbohydrates is melted at a high temperatures (>100°C) then pressed through one or more orifices (extrusion) and finally quenched to form a glass in which active agent have relatively little mobility. In general, the glass transition of encapsulates made by extrusion is between 30 and 70°C (Zuidam and Shimoni, 2010). In melt injection the melt is pressed through one or more orifices (filter) and then quenched by a cold, dehydrating solvent while in the melt extrusion the melt passes through an extruder with one or more

screws in a continuous process. The encapsulation efficiency (and the corresponding release kinetics) will depend on adequate mixing and dispersion of the flavor within the matrix as well as the presence of surface active compounds and emulsifiers. Unfortunately, the high temperatures applied in the process limits its potential use and the efficiency of encapsulation of an active compound of extruded encapsulates is relatively low (typically less than 10%), which may have an impact on their cost-in-use (Zuidam and Shimoni, 2010).

### **1.2.3.i Milling and comilling**

Milling known also as grinding, is a mechanical process largely used in various sectors (pharmaceuticals, nutraceuticals, agrochemical., aerosol, personal care, pigments, ceramic, cement, semi-conductors) to reduce the size of particles of powdered materials (Ng et al., 2011; Willart and Descamps, 2008; Morris et al., 2001).

Main objectives of this technology is to produce powders to increase the specific surface area of the solid phase for desired interactions with the neighboring phase and obtain matrices with modified technological functionalities.

In the pharmaceutical industry is the improvement of the biodisponibility and bioavailability of a substance or active compound due to the production of particles of submicron size with a higher solubility and rate of dissolution in a dissolving media, that could be attributed to an increase of the surface/volume ratio that may positively affect the solubility of a substance due to the increase of its specific surface (Willart and Descamps, 2008, Loh et al., 2015). The increased solubility permits a decrease of the concentration/dose of the active compound to achieve a specific effect. Particles of submicron size makes easy the formation of tablets thanks to the increased number of contacts among the particles that increases the formation of bridges and could also be used for the production of innovative pharma and medical products to be used in specific/target destination (e.g. aerosol, inhalation).

In the food sector milling is a technology used mainly for “macro” structural modifications by size reduction and widely applied for some low moisture food commodities and raw materials to make them more suitable for further processes (e.g.

brewing, solutes extraction) and/or formulations. In the cereals sector, milling is an important process step to produce flours used in a wide range of applications. In coffee processing, grinding is applied to roasted beans prior to the coffee brew extraction and optimization of milling conditions is critical to achieve the desired quality of the final coffee beverage.

In sugar production, different size of crystalline sucrose are obtained after evaporation of the cane syrup and its drying depending on the use and rate of dissolution desired. In the majority of the cases milling is applied to obtain particles of macro- and microscopic dimensions and the generation of submicron or nano-particles is also considered in some cases a health risk.

More recently some interest has been arising on milling processes and, in particular, ball-milling, as action able to induce phase transformation of compounds and matrices especially in the pharmaceutical sector.

Ball-milling has become, based on the processing conditions applied, a “non thermal route” of state transformation of molecular compounds (from crystalline-to-glassy) as it is carried out at relative low temperature or more in general at temperature below the glass transition (Willart and Descamps, 2008). Milled matrices present modified physical and structural properties that determine an improvement of the functionality of the final product and potential interesting encapsulating abilities when performed on a mix of two components in solid state (Lin et al., 2010, Descamps et al., 2006).

The ball-milling technologies use the friction, collision, impingement, shear or other mechanical actions in order to physically modify the crystalline structures and properties of the solid granules that is obtained by rotation of small balls within a milling chamber (Huang, et al., 2008).

Studies have evaluated the effects of the dynamic stress induced by ball milling that determine crystal-to-amorphous/glassy state transformation of compounds in solid state and a deep work on the solid state amorphisation of pharmaceuticals has been carried out by Willart and Descamps (2008) as well as other scientists (e.g. linaprazan, indomethacin, gabapentin (Lin et al., 2010; Hedoux et al., Shakhtsneider, 1997). Some

studies have been also focusing on small saccharides like trehalose (Willart et al., 2001), lactose (Willart et al., 2004) and sucrose (Tsukushi et al., 2008).

Other investigations upon ball-milling have observed, on the contrary, polymorphic transformation like in fananserine (De Gusseme et al., 2008) and in sugar alcohols like sorbitol (Willart et al., 2005) and mannitol (Descamps et al., 2007). Willart et al. (2007) highlighted a main role of the glass transition temperature ( $T_g$ ) of the liquid-solid state with respect to the milling temperature (i.e. room temperature) to explain the different effect of milling upon the development of different transformation. Some findings applied through co-milling experiments of lactose and mannitol have posed the attention to the temperature at which the process in respect to the  $T_g$  of the two component is carried out. Milling at temperatures below the  $T_g$  increases the efficiency of the crystal to amorphous transformation whereas process carried out at temperatures above  $T_g$  can lead to particle size reduction or conversion to one polymorph to another (Megarry et al., 2014, Descamps et al., 2007).

Molecular systems and matrices seem to be particularly sensitive to the effects of the mechanic stresses of milling due to:

- Presence of weak intermolecular interactions (eg. Van der Waals, hydrogen) between the molecules in the system that lead to very low elastic constant in respect to other materials like metals;
- Presence in some systems of rich polymorphic states. The transformations occurring during milling could lead to the amorphisation but also occur via a series, more or less complex, of polymorphic transformations (De Gusseme et al., 2008; Linol et al., 2007);
- In some cases the  $T_g$  of these systems are close to the ambient temperature and even limited variations of the temperature could favour the observed transformations (amorphisation, polymorphic transformations).

Besides the structural changes, thus, the milling-induced transformation could be implied in the changes of bioavailability of the compounds of interest. These transformations determine the formation of metastable systems that improve the solubility in addition to the structural changes related to the size decrease.

However, the formation of metastable systems arises issues related to the increased tendency to return to a more stable state of equilibrium upon storage with kinetics that will depend on the intrinsic and extrinsic conditions that cannot be predicted a-priori.

Studies carried out in the metallurgic sector have contributed to rationalize the origins of the amorphization and polymorphic transformations induced by milling and some models have been proposed. The following hypothesis have been, thus, suggested:

- Local melting upon mechanical stress followed by a rapid temperature quenching
- Local damage of the crystalline state that impair the structural stability of the crystal (Fecht, 1992). The system becomes stable by default accumulation created by shearing (Willart and Descamps, 2008).

Martin and coworkers (1996) by carrying out researches in the metallurgic field are also supporting the hypothesis that transitions during ball milling could take place as transitions between stationary states of “driven” alloy: shear induces some sort of disorder which is annealed by thermal diffusion (Pouchet et al., 1996).

To improve the functionality of the products obtained by the mechano-induced transformation of solids and avoid drawbacks due to their application, some developments of the technology have been carried out and are currently available.

Suspensions of nano-dimensions below the postulated grinding limit of 0.5 mm, could be obtained by applying nano-milling and by using stirred media mills in presence of surfactants. Nano-suspensions could be characterized by an increased surface area of up to 10-fold and a saturated solubility by 6-fold (Ng et al., 2011).

Co-milling represents an interesting technology based on the simultaneous milling of two or more components thereby a control of the solid-state properties of the milled powders. This technology has been used largely to prepare co-crystals or alloys which present different properties in respect to the initial components as well as inclusion complexes and solid dispersions (Watanabe et al., 2003; Ranpise et al., 2010). Milled-induced amorphization or the loss of long-range crystalline structure of solids could be

impair the stability of the amorphous phase, thermodynamically unstable that could induce re-crystallization. The latter phenomenon could occur also in long-time milling processes.

Co-milling has been used for different purposes and final matrices of different nature and functionality have been obtained so far. The milling in presence of crystalline seeds has been recognized useful to mitigate re-crystallization, while the simultaneous use of polymeric additives may increase the solubility and dissolution properties of pharmaceuticals and their final stability. Encapsulation effects have been also observed when porous materials, are used where the amorphous solid entraps the comilled solute in nano-sized channels (Ng et al., 2011).

Interesting applications in food-related matrices include those applied on mannitol-lactose and sucrose-glucose (Willart and Descamps, 2008; Megarri et al., 2014).

Recently in food sector and at more macroscopic level co-milling of olive oil and lycopene thereby an increased retention of lycopene was obtained (Bendini et al., 2015).

Cryo-milling is a specific development of the conventional milling technology applied under thermal conditions (low temperatures and, in general  $T < T_g$ ) that may favour the amorphisation effect of the high mechanical shear stresses. The equilibrium between formation of defects in the crystal structure of the initial matrix and the following thermally aided re-crystallisation of these defects according to the “alloy driven” concept of Martin and Bellon (1996) could also justify the increased milling efficiency of cryo-milling (Megarry et al., 2014).

## ***EXPERIMENTAL PART***

## 1. MATERIALS AND METHODS

### 1.1. MATERIALS

Trehalose (TRE), sucrose (SUCR) and  $\beta$ -lactose were investigated in this study while R- and S-Limonene enantiomers were used as aroma compounds. Main chemical, physicochemical and physical properties of the compounds are reported in **Table 1.1** and **1.2**.

	Trehalose	Sucrose	$\beta$ -lactose
<b>Molecular Weight</b>	342,29	342,29	342,29
<b>T melting (°C)</b>	203 (anhydrous) 97 (dihydrate)	186	202.8
<b>Tg (°C)</b>	122	75	101
<b>Solubility (water, 25 °C)</b>	68.9 g/100g	200g/100 ml	0.22 g/g
<b>logP (20°C)</b>	- 3.77 <sup>a</sup>	-3.30 <sup>a</sup>	-5.32

<sup>a</sup> Mazzobre et al. 2005

**Table 1.1.:** Chemical, physicochemical and physical properties of trehalose, sucrose and  $\beta$  lactose

	(R)-(+)-Limonene	(S)-(-)-Limonene
<b>Molecular Weight (Da)</b>	136.23	136.23
<b>T melting (°C)</b>	-74	-74
<b>Tg (°C)</b>	-136 *	-136 *
<b>T boiling (°C)</b>	175.5-176	175.5-176.0
<b>Density (20 °C) (g/cm<sup>3</sup>)</b>	0.8411	0.8411
<b>optical activity [<math>\alpha</math>]<sub>20</sub><sup>D</sup> c = 10% in ethanol</b>	+115.5±1° (SIGMA-ALDRICH)	-94±4° (SIGMA-ALDRICH)
<b>water solubility (20°C) (ppm)</b>	13.8 ppm	-
<b>logP</b>	4.23	-
<b>Vapour pressure (Pa, 20°C)</b>	190	190

\* experimental data from this work

**Table 1.2. :** Chemical, physicochemical and physical properties of (R)- and (S)-limonene

Three different samples of trehalose were used:



- Di-hydrate trehalose (Sigma, St. Louis, MO, USA; purity >99%), for the study of the liquid-vapor partition of the aroma compounds and for the preparation of freeze-dried and spray-dried samples;
- Anhydrous trehalose (ACROS, Geel, Belgium, purity >99%) for all the different milling and co-milling experiments;
- Di-hydrate Trehalose (TREHA® Trehalose) from Cargill for surface tension measurements.

R- and S-Limonene were provided by Sigma (Sigma, St. Louis, MO, USA). Anhydrous  $\beta$ -lactose ( $\leq 30\%$   $\alpha$ -anomer basis,  $\geq 99\%$  total lactose) and sucrose were provided by Sigma (Sigma, St. Louis, MO, USA).

## 1.2 METHODS

### 1.2.1. SAMPLES PREPARATION PROCEDURES

#### 1.2.1.1 Spray-Drying

To obtain low-moisture, amorphous powders two different preparation procedures of the initial feeding solution were applied depending on the presence/absence of limonene:

- Trehalose solutions (20, 40% w/w): dihydrate trehalose was dissolved in distilled water at 20°C and kept under agitation for 2 h until the complete sugar solubilization.
- Emulsions of limonene in trehalose solutions. Due to the low water solubility of limonene, the aroma was preliminarily dispersed in trehalose solutions at different concentration by a two-steps homogenization procedure to obtain microemulsions according to Kaushik and Roos (2007). Briefly, aliquots of water, trehalose and limonene at the defined concentration were initially poured in a glassy jar and immediately closed with a hermetical cap and kept under agitation for 20 min at 20 °C. This suspension was then roughly homogenized by a rotor-stator device (Ultra-Turrax, yellow line, DI25 basic) for 30 s at 13.500 rpm by inserting the rotator into the glassy jar and to avoid

aroma loss the rotor and the jar was covered by an adhering polyethylene film and a further aluminium film. Thereafter the pre-emulsion was processed by a high pressure homogeneizer (Panda PLUS 2000, GEA Niro Soavi, Parma, Italy) at a pressure of 750 bar. Under these conditions, based on the hydrophobic properties of (R)-LIMO, oil-in-water (o/w) emulsions were obtained.

The emulsions were collected and immediately subjected to spray-drying. Samples of the emulsions were taken for dispersion degree and stability evaluation.

- *Spray-drying*

Solutions of trehalose and emulsions of (R)-Limo in trehalose solutions at different saccharide and limonene concentration made as above described were spray dried using a Spray Dryer B-90 (Buchi Labortechnik AG, Postfach, CH) using the following operating parameters: atomizer flow rate 100 l/min; pressure: 45 mbar; machine setting: aspirator 100 % and feed rate: 1 ml min<sup>-1</sup>; porosity of the atomizing membrane: 7 mm. Spray-drying was carried out on batches of initial sugar solutions or limonene-based sugar emulsions of 250 ml.

Two series of samples were prepared with different purposes:

Series 1. Samples to investigate the compositional properties (trehalose and limonene concentration) at constant process conditions (T inlet: 120°C). These spray-dried samples have been analyzed both just after processing and during storage at low Relative Humidity (RH< 10%) (**Table 1.3**).

Series 2. Samples to investigate both compositional properties of the initial sugar solution and of the limonene emulsion in the trehalose –based water phase as well as process conditions (**Table 1.4**)

Sample	Concentration	
	Trehalose (% w/v)	(R)-Limonene (ppm)
Trehalose	20	-
	40	-
Trehalose + R-limonene (emulsion)	20	2500, 5000
	40	5000

**Table 1.3.:** Composition of initial feeding systems used for spray-drying experiments (series 1)

Initial solution/dispersion composition		Atomisation temperature (°C)
Trehalose (% w/v)	Limonene (ppm)	
20	2500	110
20	10000	110
20	10000	160
20	2500	160
20	6250	145
20	6250	145

**Table 1.4.** Composition of initial feeding systems used for spray-drying experiments (series 2)

The spray-dried powders just after their collection have been sampled for residual moisture content analysis while the remaining part was poured in Petri plates as thin layers within a desiccator over phosphorus pentoxide (0% RH) at 15°C for the removal of the residual moisture not removed by the drying process. Thermal analyses were carried after 10 days of storage as above described.

Monitoring of the moisture loss of the just made samples during storage was carried out and under the conditions used, samples analyzed had moisture content lower than 2.7 %.

#### 1.2.1.2. Freeze-Drying

Freeze-drying was carried out by using a benchtop freeze-dryer (model UNITOP 400 L, Virtis, Gardiner, NY, USA). About 150 mL of 20% w/v trehalose solution and of microemulsions of limonene in 20% w/v trehalose solution, in two separated Petri plates were cooled to -45 °C and held for 2 min and then subjected to reduced pressure (100 mTorr). Temperature was then slowly increased to -30 °C, and primary drying was carried out for 38 h. The temperature was gradually increased to 50 °C over a 24 h period, and secondary drying was carried out for 31 h. This was followed by additional drying at 20 °C for 24 h.

#### 1.2.1.3 Milling and co-milling

Ball milling was carried out at room temperature (RT) by using two high energy planetary micro-mill equipment both provided by Fritsch (FRITSCH, Idar-Oberstein,

Germany) one located at the University of Lille (FR), the other at the University of Teramo (IT).

In particular: the following apparatus were used:

- Pulverisette 7, equipped with a  $ZrO_2$  milling jars of  $45\text{ cm}^3$  with seven balls ( $\varnothing=15\text{ mm}$ ) of the same material (University of Lille).
- Pulverisette 6 equipped with a  $ZrO_2$  milling jars of  $80\text{ cm}^3$  with eighteen balls ( $\varnothing=15\text{ mm}$ ) of the same material (University of Teramo).

In all experiments samples were carried out starting from aliquots of 1 g of anhydrous solid (trehalose, sucrose or  $\beta$ -lactose) placed in the planetary mill chamber; a corresponding ball/sample weight ratio 49:1 was obtained. To avoid losses of the volatile, the addition of the limonene according to the desired weight was made by using a microsyringe directly in the jar containing the sugar that was then immediately closed. The jars rotate with the same rotation speed  $\Omega$  but on the opposite direction to the disc on which they are fixed. The rotation speed of the solar disk was kept constant independently on the apparatus used and equal to 400 rpm.

To avoid any overheating of the samples, milling periods of 15 min were alternated with pause periods of 5 min and reported values of milling time correspond to the sum of the effective durations (excluded the rest/pause time).

Both apparatus are kept in rooms where the external temperature as well as that of the jar was conditioned and kept constantly below  $25^\circ\text{C}$ .

Just after milling samples were removed from the jar in a low relative humidity environment to avoid moisture uptake, inserted in vials and located in a desiccator under  $P_2O_5$  for at least 24 h prior to analysis at room temperature.

For sake of clarity, samples made just with the sugar are named as “milled” while those made by milling together sugar and limonene are named as “comilled”.

Independent variables of milling/co-milling investigated in this project were:

- presence/absence of limonene
- milling time (2, 4, 6, 9, 12, 16, 18 h)
- limonene concentration (2.7, 5.4, 11.0, 18 % on weight basis)

- limonene enantiomer (R-, S-)

Data reported are the result of at least two repetitions of the milling/co-milling process carried out in different times.

### **1.2.2. SAMPLES STORAGE PROCEDURES FOR STABILITY EVALUATIONS**

Differently processed spray dried, milled and comilled samples prepared by using trehalose, sucrose and lactose with or without limonene were poured just after preparation in glassy vials of 2 ml, hermetically closed and stored at 20°C within a glass desiccator where at RH % < 10 was kept by silica gel.

Series of milled and comilled trehalose and spray-dried samples differently prepared have been kept for 6 months. Analyses to evaluate changes in the thermal properties and aroma retention have been carried out after 2 and 6 months.

### **1.2.3. ANALYTICAL AND INSTRUMENTAL METHODS**

#### **1.2.3.1. *Surface tension measurements***

The surface properties of (R)-Limonene were investigated by measuring the surface tension in water and in sugars solutions by a KSV Attension Sigma 700/701 tensiometer (Biolin Scientific Oy, Espoo, Finland), equipped with a Wilhelmy plate. Sample was inserted in the vessel (diameter: 70 mm) that is then moved up so the sample just comes into contact with a thin platinum plate. Samples analysed were:

- limonene in water at different concentrations (concentration range 2.5 – 50 ppm)
- limonene at different concentrations (concentration range: 5-20 ppm) in 10 and 40% trehalose or sucrose solutions
- 10 and 40% trehalose or sucrose solutions

Water was taken as reference and its surface tension determined daily before the start of the analysis. Measurements (20°C) were made over time and data reported are

those collected after 30 min. For some samples measurements up to 48 h hours were carried out.

For each sample, at least 2 repetitions from three different samples made in different times were collected (2 x 3 = 6 data).

### **1.2.3.2. Dispersion degree of limonene in water and saccharide solutions**

Particle size measurements of limonene droplets in water and sugar solutions were carried out by a laser diffraction granulometer Mastersizer Hydro 3000 (Malvern Instrument, Malvern Worcestershire United Kingdom), according to Kaushik & Roos (2007). The sample was dispersed in distilled water at 2000 rpm until an obscuration rate of 12 % was reached. Optical properties were defined by using a refractive index 1.46 and absorption 0.00.

Droplet size measurements are reported as the volume-weighted mean diameter  $D_{4,3}$  or De Brouckere diameter ( $\mu\text{m}$ ) and the volume-surface mean diameter or Sauter diameter  $D_{3,2}$ . The former is more sensitive to changes in particle size involving destabilization processes and used to evaluate emulsions structural stability (flocculation or coalescence); the latter is inversely proportional to the specific surface area of droplets and provides a measure of the mean diameter where most of the particle fall (Relking and Sourdet, 2005).

Analyses were performed:

- just after emulsification to evaluate dispersion degree
- after 24 h of storage (in hermetically closed vials, 20°C, dark), to evaluate emulsion stability.

Data reported are the average of ten readings made on each of two independently prepared emulsions.

### **1.2.3.3 Thermal analysis**

Thermal analysis has been carried out on low moisture/dried samples differently processed and stored by Differential Scanning Calorimetry (DSC). Different equipment have been used:

- Perkin-Elmer DSC 7 (Differential Scanning Calorimetry, DSC 7, Perkin Elmer, Boston, MA) equipped with cooler
- DSC Q1000 of TA Instruments

Samples of powders (4-6 mg) have been weighted in aluminum pans and either left open (container without cover) or sealed with the standard pierced aluminum cover (50 micron diameter hole centered in the cover). The experimental procedure was carried out according to the procedure suggested by Willart et al. (2001) as some water has been found to be unavoidably present in samples due to either strongly bound water not removed by the drying process (spray-drying, freeze-drying) or caught by the samples during the milling process. This procedure made easier the experiments and avoided the occurrence of phenomena induced by the residual water.

Temperature scans were carried out in the following ranges:

- 20°C to 230°C (270°C, for lactose)
- -196°C to 50°C (for evaluation of the thermal behavior at low temperature), at scan rate of 5°C min<sup>-1</sup>.

The following instrumental scanning conditions have been used:

- From 20°C to 230°C at 5 min<sup>-1</sup> (1<sup>st</sup> scan)
- From 230°C to 20°C at 50 min<sup>-1</sup> (cooling step)
- From 20°C to 230°C at 5 min<sup>-1</sup> (2<sup>nd</sup> scan).

During analysis the calorimeter head was flushed with nitrogen at a flow rate of 40 ml/min.

In all cases temperature and enthalpy readings were calibrated using pure indium at the same scan rates used in the experiments. For measurements carried out at temperature below 0°C, DSC was calibrated for temperature using n-hexane (melting point, -95.0 °C) and water (melting point, 0.0 °C).

For each sample, at least two repetitions of the scan have been made.

#### **1.2.3.4. Time Domain Nuclear Magnetic Resonance (TD-NMR)**

The free induction decay curve of each sample (ca. 500 mg) was acquired using a Bruker "The Minispec" PC/20 spectrometer (Bruker Biospin GmbH, Rheinstetten,

Germany) operating at 0.47 T and a resonance frequency of 20 MHz. Samples were placed into 10-mm diameter NMR tubes and analyzed at 25 °C. The dwell time was set at 0.4 μs, the acquisition time adjusted to 1 ms and 128 scans were acquired with a recycle delay of 5 s. The resulting decay signal was fitted using the following equation (1):

$$S(t) = A_1 \exp\left(-\frac{a^2 t^2}{2}\right) \frac{\sin bt}{bt} + A_2 \exp\left(-\frac{t}{T_{2,2}^*}\right) + A_3 \exp\left(-\frac{t}{T_{2,3}^*}\right) \quad (1)$$

where  $A_i$  is the amplitude of each component  $i$  and  $T_{2,i}^*$  its spin-spin relaxation time,  $b$  is the half-width of a rigid proton spectrum and  $a$  is the standard deviation of the Gaussian line-shape of the rigid proton spectrum (Kumagai et al., 2002). Two exponential decays were used to fit the relaxations of the mobile protons and results are expressed as the relaxation time of the most mobile protons (Total  $T_{2}^*$ ) and the average  $T_{2}^*$  value of the two exponential decays.

In addition, the second moment  $M_2$  describes the strength of the dipolar interactions and gives information about the mobility of the immobile protons. It was calculated, according to Buitink et al. (2000), using the fit parameters  $a$  and  $b$  by the equation (2):

$$M_2 = a^2 + \frac{b^2}{3} \quad (2)$$

#### **1.2.3.5 Limonene release/retention and partition coefficient in sugar solutions**

Liquid-vapor partition coefficient ( $k$ ) of the limonene was determined using the Phase Ratio Variation method (PRV), a static and indirect method, based on the relationship between  $k$  and  $\beta$ , defined as:

$$\beta = \frac{V_g}{V_m}$$

where  $V_g$  is the gas volume and  $V_m$  is the matrix volume.

This method has been successfully used by several authors for the determination of partition coefficients of volatile compounds in mixture and complex matrices (Martuscelli et al., 2008; Savary et al., 2006; Jouquand et al., 2004).



This method involves the gas-chromatographic analysis of a standard volume of head-space taken inside a vial hermetically sealed, in which increasing volumes of matrix (decreasing  $\beta$ ) were added.

Ette et al. (1993) determined the following equation to calculate  $k$  coefficients:

$$\frac{1}{A} = \frac{1}{f_i C_m} k + \frac{\beta}{f_i C_m}$$

where:

$A$  = gas-chromatographic peak of the analyte in equilibrium;

$f_i$  = proportionality factor;

$C_m$  = initial concentration of the analyte in the matrix.

The only variable of the equation are  $A$  and  $\beta$ . The previous equation corresponds to a linear equation of the type:

$$\frac{1}{A} = a\beta + b$$

where:

$$a = \frac{1}{f_i C_m}$$

$$b = \frac{1}{f_i C_m} k$$

$k$  coefficient ( $= a/b$ ) is calculated from  $a$  and  $b$  values, obtained putting in a graph the reciprocal of the peak area values vs  $\beta$ .

In this study, for the calculation of the partition coefficient, increasing volumes of each sample (50, 100, 250, 500, 1000  $\mu$ L) were added in 20 ml-glass vial and hermetically sealed. For each volatile compound, the  $k$  value was calculated as ratio between the mean of the slopes,  $m$ , and the mean of the intercepts,  $q$ , of the line. It was established that linearity was observed when  $R^2 > 0.80$ .

GC analysis of the head-space of each sample for the determination of  $k$  coefficient was performed with a 6890N gas-chromatograph coupled with a flame ionization detector (FID) (Agilent Technologies, Santa Clara, United States), and equipped with a HP7694 headspace auto sampler (Agilent Technologies, Santa Clara, United States). Analysis was performed on 1 ml of the vapor phase in equilibrium in the head-space of each vial and injected in a DB-5 column (60 m length, 530  $\mu\text{m}$  – 5.00  $\mu\text{m}$  film thickness) (J&W, Agilent Technologies), in split-less mode. Each vial was equilibrated at 37 °C during 60 min in the static headspace sampler.

#### **1.2.3.6. Limonene retention in low moisture sugar matrices**

The evaluation of the limonene in the differently prepared saccharide samples was carried out to determine the entity of retention in the differently processed and amorphized matrices as well as the loss of the aroma during storage.

Due to the type of processes applied and the characteristics of the samples obtained (dried, powder-like) presence and quantity of limonene was evaluated two different steps of extraction were carried out to evaluate the aroma present both at the surface of the sample (as residual of the process or adsorbed) and that entrapped/encapsulated in the sample.

##### **1.2.3.6.i Determination of the limonene on surface**

The amount of limonene present on the surface of the low moisture-samples obtained by co-milling, spray-drying and freeze-drying and/or by mixing just after preparation or after storage was determined by gas-chromatographic analysis after extraction as described by Kaushik and Roos (2007), with some modifications. An aliquot of 500 mg of powder was mixed with 10 ml of hexane (HPLC Grade, Sigma Aldrich) in glass bottles (sealed with teflon coating) and agitated with a “Rota mixer” for 5 min. Powder particles were separated from hexane by centrifugation at 3500 rpm for 5 min. The supernatant has been transferred into hermetically closed vials of 2 ml and analyzed by the GC-MS method as described in 2.2.2.5.iii.

#### **1.2.2.6.ii Determination of the encapsulated/entrapped limonene**

The powders obtained after washing the surface with hexane as described in section 5.3.2. were used to determine the amount of encapsulated limonene according to Kaushik and Roos (2007), with some modifications. An aliquot of 500 mg of powder was dissolved in 10 ml of demineralized water and then 5 ml of hexane was added, followed by mixing with a "Rota mixer" for 1 min and heated at 40°C for 20 min with intermittent mixing. The bottles were cooled to room temperature and hexane was separated from aqueous phase by centrifugation at 3500 rpm for 5 min. The supernatant has been transferred into hermetically closed vials of 2 ml and analyzed by the GC method described in section 2.2.2.5.iii.

#### **1.2.3.6.iii GC-MS analysis**

Extracts (2.2.2.5i, 2.2.2.5ii) were analyzed by GC-MS analysis. A Perkin Elmer gas chromatograph 580 coupled with a Clarus SQ 8 S detector (Perkin Elmer, Boston, MA) equipped with a RXI-5ms column (length 30 m, 0.25 mm internal diameter and 0.25 µm film thickness; Restek Bellefonte, PA). The following settings were used: sample volume: 0.5 µl; injection temperature: 250°C; split ratio 1:10; gas carrier: Helium; He flow rate: 1 ml min<sup>-1</sup>; oven profile: starting T: 60°C; 60°C kept for 1 min, then from 60 °C to 250 °C at 10°C/min and kept at 250°C for 1 min.

Calibration curve of limonene was performed before every quantification analysis.

#### **1.2.3.7. Morphological and microstructural properties**

Particle morphology and microstructural properties of the amorphous, low moisture samples was investigated by Scanning electron microscopy (SEM) by using a Field Emission SEM (LEO 1525 equipped with a GEMINI column, ZEISS, Germany). Samples were prepared depositing powders onto an aluminum specimen stub covered with a double sided adhesive carbon disc. Samples were sputter coated with chromium (8 nm thickness) prior to Imaging (Quorum Q150T ES East Grinstead, West Sussex, UK).

#### **1.2.3.8. Water content (Karl Fisher method)**

Moisture content in the dried powders was determined by Karl Fischer titration by direct injection using a Mitsubishi model CA-06 moisture meter (Mitsubishi Kasei Corporation, Tokyo, Japan). The powders were dissolved/dispersed in 2.5 mL anhydrous formamide and 1 mL of the solution was injected. Titration checks were made by injecting water standards (MitsubishiMC02020) at several time intervals between injections. Blank corrections were applied. Standard deviation from replicate measurements is reported in R&D session for each sample.

## **RESULTS AND DISCUSSION**

## **1. INTERFACIAL, THERMODYNAMIC ASPECTS AND MASS TRANSFER OF LIMONENE IN CARBOHYDRATE SOLUTIONS**

In a preliminary part of the project some physico-chemical properties related to the hydrophobicity or hydrophilicity of limonene in water (binary systems) and in carbohydrate solutions (ternary systems) were investigated. The study was mainly focused on trehalose solutions and where of interest, sucrose solutions were taken as reference.

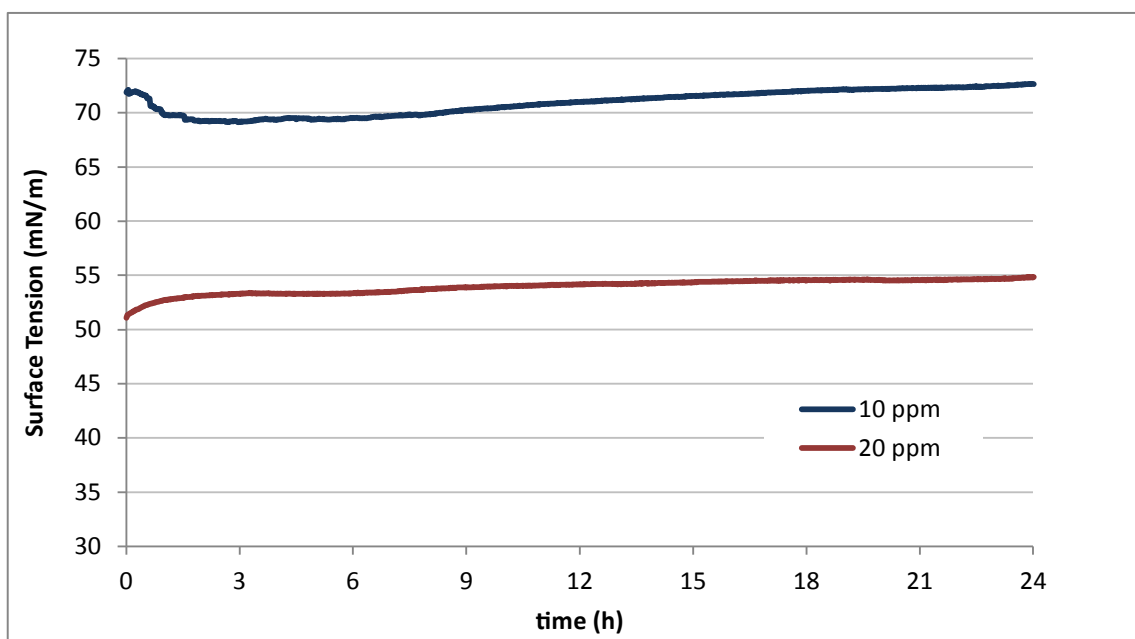
Our attention was thus focused on the surface tension of limonene in water and disaccharide solutions and on the limonene release and the liquid-vapour partition coefficient in aqueous systems at different solute concentration. The determination of the amphiphilicity of the limonene in water and saccharide solution and its tendency to partly in the vapour phase could allow to better evaluate the impact of the spray drying on the limonene retention as well as the following stability over storage

The thermodynamic aspects of the liquid-vapour partition in water and sugar solutions were also investigated to better characterize the effects of the presence and concentration of disaccharides in aqueous systems that could affect the retention during the heating and rapid undercooling occurring during spray drying.

### **2.1. Surface Tension (ST)**

Surface tension has receiving increasing attention due to its role in the mass-transfer phenomena and processes occurring at air-matrix interface that may affect the release/retention of volatile compounds during processing and storage. Mass transfer coefficients at the interface of volatile compounds like diacetyl and heptan-2-one resulted affected by the surface organization-reorganisation of the solution-air interface (Harrison & Hills, 1997). Secouard et al. (2006) recognized the role of surface tension as index of intra- and intermolecular non-polar interactions of xantan molecules on the release of limonene below and above the critical micellar concentration of the polysaccharide.

Surface (air/water) and interfacial (oil/water) tension play also a main role in structuring dispersed and colloidal systems like foams and emulsions by affecting bubble or droplet physical properties and their stability over processing and storage. In our study limonene, a relative apolar volatile compound, has been taken as model of volatile compound for the effects on the physical properties of glassy disaccharide matrices obtained by different processing approaches. Spray drying has been taken as process of amorphysation starting from a high moisture carrier solution containing only limonene and the saccharide at different concentrations without the addition of any additional surfactant agent. Spray-dried powder particles may present physical properties (e.g. size, shape, etc.) depending on the specific surface/interfacial properties of the starting carrier dispersion and process conditions. The determination of the surface tension properties of the limonene-saccharide aqueous systems could, thus, allow a better understanding of the physical properties of the corresponding spray-dried systems as well as their ability to retain/encapsulate limonene.



**Figure 2.1.** Surface tension kinetics of aqueous solution of (R)-LIMO at 10 and 20 ppm (25°C)

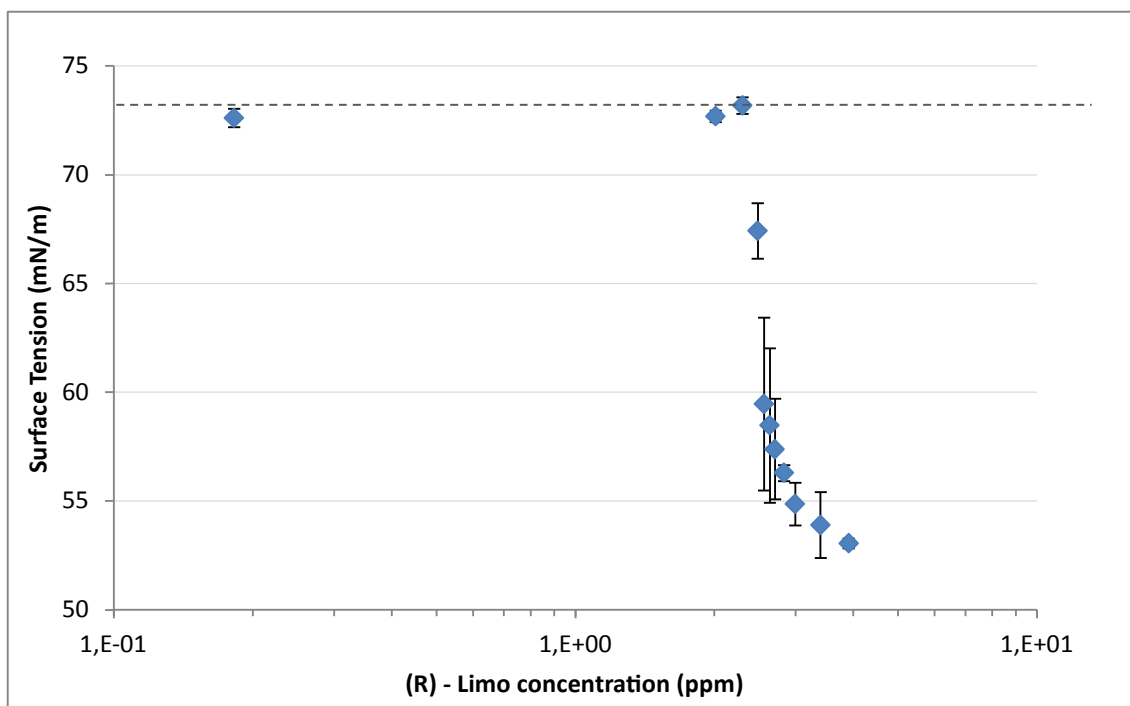
Preliminary experiments were carried out to evaluate the effect of the adsorption time of (R)-LIMO at air/water interface and in **Figure 2.1** the kinetic of the surface tension in water at two different aroma concentration is reported. The concentrations of 10 ppm and 20 ppm were chosen being one above and the other below the solubility of the (R)-LIMO in water (25°C). The change of ST over time is clearly different depending on the (R)-LIMO concentration.

At 10 ppm an initial decrease of the ST could be observed until 3 h at which an increase occurs and after 24 h the ST value is close to that of starting water. On the contrary when (R)-LIMO is present in concentration above its solubility limit, surface tension immediately starts from a significant lower value in respect to water that tends to increase to a limited but significant extent in the following 2-3 h and less in the following 20 h by keeping values significantly lower than that reached after the same time by the solution of 10 ppm (R)-LIMO.

In this study ST of (R)-LIMO has a relative importance as it could affect the structuring of amorphous sugars during spray drying. In this case the formation of an “aroma emulsion” in the disaccharide emulsions was required due to limited solubility of (R)-LIMO. No additional surface active compounds were added (e.g. emulsifiers) and or cosolutes like maltodextrins or proteins able to contribute to the formation during spray drying of stable droplets. The ability of the limonene as small molecule to reach quickly the rapidly forming interface of the oil (Limo) /water or sugar solution and to stabilize for a sufficient time was, thus, a critical point.

For the formation of dispersed systems like emulsions, the ability to quick reach the lipid/water interface is required during the rapid formation of the transient and increasingly formed interfaces during emulsification. Thus, further measurements of ST were carried out at different (R)-Limo concentrations for a maximum time of 30 min of adsorption/equilibration. The variation of the surface tension of (R)-Limo versus its log C in water (20°C) is shown in **Figure 2.2**.





**Figure 2.2:** Plot of the surface tension of (R)-Limo as a function of its concentration in water

At low concentrations, surface tension results very close to that of water while above 10 ppm it varies significantly, by decreasing, up to a concentration of 17-20 ppm where increasing aroma concentration do not determine main changes at the ST value remains practically constant by reaching a plateau value of  $53 \text{ mNm}^{-1}$ . To our knowledge, no data are available in the scientific literature on the change of the surface tension of limonene in water and/or in other solvents.

The trend in the ST change here reported is similar to that of small-molecule surfactants characterized by an amphiphilic behavior due to the presence in the moiety of both a hydrophobic and a hydrophilic part. (R)-Limo at very low concentrations dissolves readily in water while no main effects on the surface tension of water occur while above 10 ppm a significant and decreasing linear effect with the increased aroma concentration on the  $\gamma$  value occurs likely due to the higher adsorption of the molecules at the air/water interface with a decrease in free energy. The further change of the trend above 17-20 ppm in respect to the previous one could be due to the formation of association colloids (i.e. micelles, bilayers, vesicle) that may

occur in small molecule surfactants above a certain concentration defined as Critical Micelle Concentration (CMC). The hydrophobic part of the molecule tends to closely pack to form structures able to diminish their contact with water with a consequent decrease of the free energy while it decreases the mixing entropy (Walstra, 2003). Micelles could have different size and shape and contain 50 to 100 molecules in the case of small molecules surfactants. They are also dynamic structures and molecules could move in and out in a span time of 10  $\mu$ s (Walstra, 2003).

Surface tension could be fitted by equations that model its trend versus concentration to determine CMC of surface active molecules as well as by extrapolation techniques. By using the latter approach to our data, the CMC of (R)-limonene in water resulted equal to 15.5 ppm value that is slightly above the limit of solubility of this aroma compound (13.8 ppm).

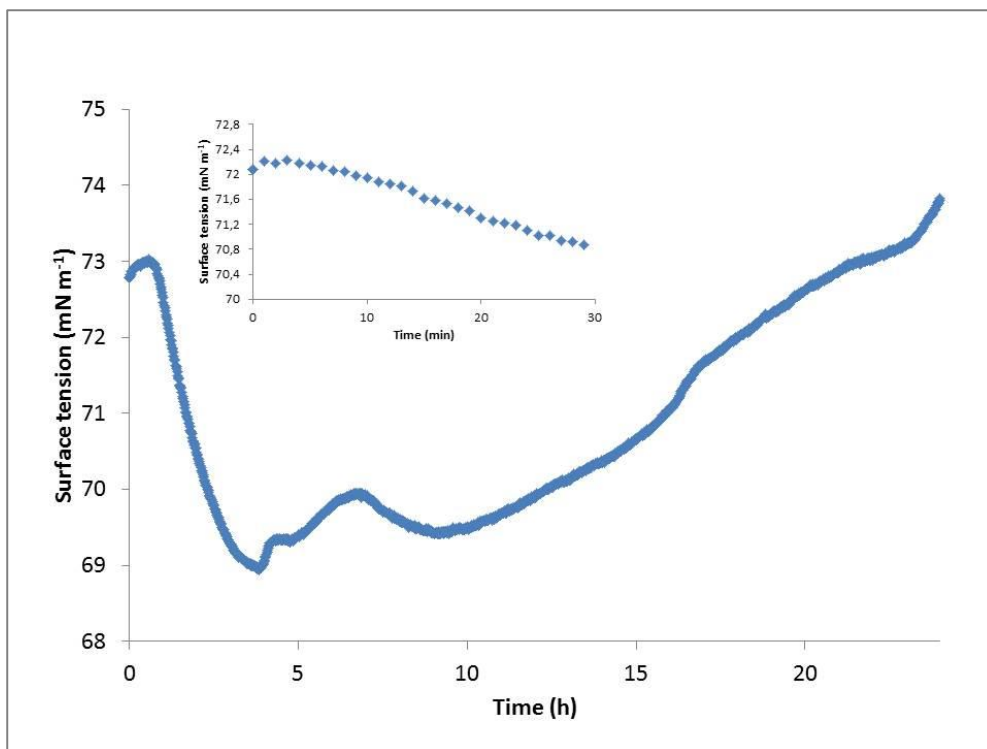
Surface tension of (R)-LIMO and disaccharide solutions and their mixtures at the same adsorption time was then determined to evaluate the effect of the water phase composition and, in particular, of the nature and concentration of the sugars. Trehalose and sucrose solutions at 10 and 40 % were investigated and in **Table 2.1**. the obtained values (20°C) after 30 min of equilibration time are reported.

Values of ST of the sucrose and trehalose solutions at both 10 and 40% resulted lower than that of water and the lower  $\gamma$  was found for the more concentrated solutions. It is generally recognized that hydrophilic solutes like sugars determine an increase of the surface tension of water and data confirming this behavior are reported by several authors (Aroulmoji et al., 2004; Docoslis et al. 2000; Kita et al., 1994; Matubayasi et al., 2006). The higher surface tension of aqueous solutions of sugars and osmolites including TRE has been also associated to their effects as stabilizers of proteins against thermal denaturation (Kita et al., 1994; Lin & Timasef, 1996). This is caused by the elevated free energy of cohesion between the electron-acceptor and electron-donor sites of the molecularly dissolved sugar molecules as a result of hydrogen bonding. However, according to Docoslis et al. (2000) the formation of a more concentrated layer of the solute than the bulk solution and the solubility limit along with the formation of micelle-like structures may cause a depletion layer at the air/water

interface and loss of hydration that may cause a drastic apparent decrease in the measured surface tension of the aqueous solvent. Aroulmoji et al (2004) found an increase of ST from 73.5 to 74.5  $\text{mN m}^{-1}$  in sucrose solutions at increasing solute concentration up to 0.75 M while Docoslis et al. (2000) found an experimental increase of 0.55 and 2.1  $\text{mJ m}^{-2}$  in respect to the ST of water for solutions of sucrose at 10 and 40% sucrose. Kita et al. (1994) found an increase of trehalose similar than that of sucrose and equal to 1.34  $\text{dyne cm}^{-1}/\text{mol}$  and Hutteau and coworkers (1998), a value of 73.9 for a 10 % sucrose solution.

On the contrary, some papers report values of surface tensions of sugars lower than that of water. In particular ST data for 10 % TRE solutions obtained in our study are similar to those reported by Schmidt et al (2011) that by a standard tensiometer plate determined a  $\gamma$  value solution equal to  $69.42 \pm 0.03 \text{ mN m}^{-1}$ .

To better clarify the discrepancy of our ST data obtained after 30 min in respect to the main scientific finding, surface tension measurements of the sugars solutions for longer time and up to 24 h was carried out in order to better evaluate the dynamics of the molecular adsorption of the saccharides molecules at the interface and the achievement of a pseudo-equilibrium state. Under these conditions surface tension of the TRE solutions (10 and 40%) showed to change as a function of time with a no linear trend (10 % TRE solution, Figure 2.3.), starting with an initial decrease in the first 3-5 hours followed by a progressive increase in the following time to reach, after 24 h a final value of 73.8  $\text{mN m}^{-1}$  which is more consistent with data reported in the literature. For the 40 % TRE solutions, longer time of equilibration were required to achieve ST values similar to those reported in the literature (Kita et al. , 1994) and in agreement with the scientific findings about the role of the non-ionic organic solutes on the surface tension (Docoslis et al., 2000).



**Figure 2.3.** Change of surface tension of 10% TRE solution (20°C)

As regards the ST of the ternary systems made of (R)-LIMO, trehalose or sucrose solutions (**Table 2.1**) in general the increasing concentration of the volatile in the saccharide solution determine a decrease of the ST whose entity increased at increasing aroma concentration. The trend of the ST at increasing limonene concentration was different than that observed in water that started from a ST value of the lower aroma concentration that that found in water showed a progressive decrease with a less steep change of ST in the range of 10-15 ppm concentration where differences due to saccharide type and concentration of the solution were observed. However, at 50 ppm of limonene, values of ST observed were non statistically different than those observed in water for all the sugar solutions under study. It is known that cosolutes like sugars and other polyols, alcohols and salts may affect the critical micellar concentration of nonionic surfactants by decreasing it due to chemical and physicochemical effects of the solute present in the aqueous phase that may affect the solubility of the surfactant agent. The decrease has been attributed to

changes in solubility and/or in the solvation state of the amphiphilic molecule (Sagitani, 1988; Ueda et al., 1980; Gratzner and Beaven, 1969). A decrease of the solubility of limonene in sucrose solutions have been observed by Massaldi and King (1973) with a saccharide concentration effect. The latter authors by a gas-chromatographic method observed a linear and decreasing change of the solubility d-limonene at 25°C that from 13.8 ppm (water) reaches a value of 12.5 ppm in sucrose solutions at 60%wt. No data are, however, available for solubility of limonene in other saccharide solutions and investigations are currently ongoing.

In order to better compare the effects due to the presence of (R)-LIMO, ST results have been used to compute the surface pressure  $\pi = \gamma_0 - \gamma$ , where  $\gamma_0$  is the surface tension of the solvent (either water or sugar solution at the data concentration) and  $\gamma$  is the surface tension of the sample ((R)-LIMO aqueous solution or (R)-LIMO+disaccharide aqueous solution/mixture) and results are shown in **Figure 2.3**.

[LIMONENE] ppm	Water	TREHALOSE (w/v)		SUCROSE (w/v)	
		10%	40%	10%	40%
0	72.61 ± 0.41 <sup>a</sup>	69.65 ± 2.08 <sup>ab</sup>	67.02 ± 3.37 <sup>ab</sup>	71.37 ± 0.41 <sup>a</sup>	62.27 ± 0.65 <sup>a</sup>
7.5	72.70 ± 0.26 <sup>a</sup>	61.56 ± 2.03	61.21 ± 0.29	59.71 ± 0.82 <sup>b</sup>	59.24 ± 0.99 <sup>b</sup>
10	73.19 ± 0.38 <sup>a</sup>	59.12 ± 2.41 <sup>c</sup>	54.38 ± 2.17 <sup>d</sup>	58.54 ± 3.67 <sup>b</sup>	56.41 ± 2.46 <sup>cd</sup>
15	57.38 ± 2.31 <sup>a</sup>	57.02 ± 1.38 <sup>a</sup>	52.52 ± 1.40 <sup>b</sup>	54.81 ± 2.68 <sup>b</sup>	47.57 ± 2.08 <sup>c</sup>
20	54.86 ± 0.98 <sup>a</sup>	51.15 ± 2.61 <sup>ab</sup>	46.84 ± 3.09 <sup>bc</sup>	50.59 ± 1.55 <sup>b</sup>	46.28 ± 1.35 <sup>bc</sup>
50	53.05 ± 0.23 <sup>a</sup>	51.03 ± 1.21 <sup>b</sup>	n.d.	51.00 ± 1.70 <sup>b</sup>	n.d.

<sup>a-d</sup>: different letters means significant difference ( $p < 0.05$ ) of the means in the same row

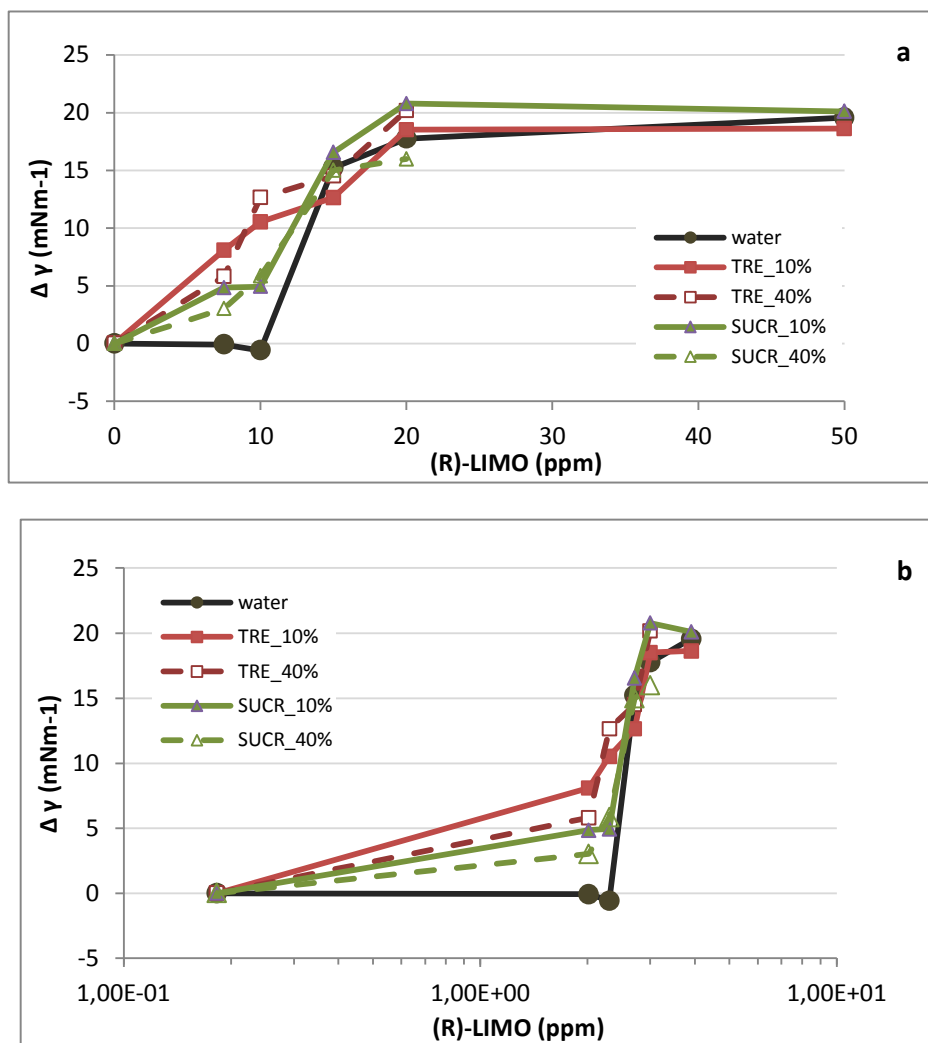
**Table 2.1:** Surface tension of (R)-Limo at different concentration in water and in TRE and SUCR solutions at 10 and 40%w/v. Data reported correspond to a 30 min equilibration time.

Surface pressure  $\pi$  is an index of the entity of the amount of adsorbed amphiphilic material at the air/water interface and the relationship between the amount of bulk and adsorbed amphiphilic material and the surface pressure is given by the Gibbs adsorption isotherm:

$$d\pi = -d\gamma = RT \Gamma d \ln A \approx RT \Gamma d \ln C$$

where  $\Gamma$  is the surface excess concentration of the amphiphilic material (i.e. amount adsorbed per unit interfacial area),  $C$  is its concentration in some dilute systems,  $T$  and  $R$  are the absolute temperature and gas constant, respectively.

It could be noticed that (R)-LIMO at low concentrations up to its solubility limit (and CMC) shows a higher  $\pi$  in TRE solutions than in the SUCR ones. Above a concentration of 20 ppm of the volatile compound no significant differences were observed in the surface pressure in both water and saccharide solutions.

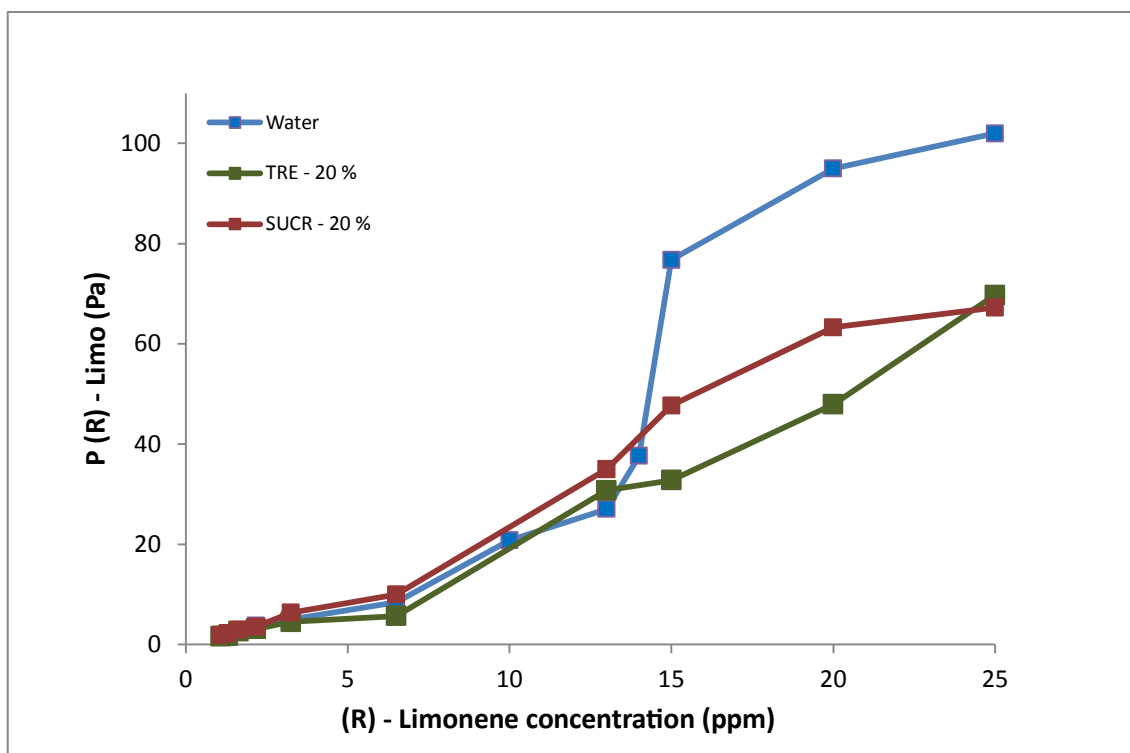


**Figure 2.3.** Surface pressure of (R)-LIMO-water and (R)-LIMO- TRE or SUCR solutions (10 and 40% w/v) as a function of (R)-LIMO concentration (a) or of  $\ln C$  (b). Data reported are referred to 30 min of equilibration time of the air-water/solution interface.

With the premise that these data are referred to a 30 min equilibration time (i.e. do not correspond to a steady value), the differences observed between TRE and SUCR in affecting the surface tension of the limonene systems could be due to their different hydrophilicity/hydrophobicity and hydration behavior of the saccharides in water as well as their physical properties (e.g. viscosity) that may differently affect the kinetics of the limonene to reach the air/water interface.

Although many studies have been carried out on trehalose-water and sucrose-water systems, a coherent picture of their behavior as a function of concentration and temperature is still difficult to be captured. For example, TRE-water system is often claimed to be characterized by a higher value of hydration number than sucrose (Branca et al., 2001), but this effect (destructuring) changes with concentration and temperature. Indeed, when a solute molecule is in an aqueous environment, its functional groups must interact with the inherent structural requirements of the surrounding water, and its presence can impose a structuring pattern on the adjacent solvent molecules which differs from that of pure bulk water and depends on solute concentration. All of the peculiar water properties have long been directly or indirectly ascribed to its unique hydrogen bonding capacity. As a consequence, solvent structuring has been recognized to be of critical importance in determining the properties of all aqueous solutions. It has been very difficult altogether to directly determine in practice the nature of this structuring. The organization of solvent water molecules around a particular solute will in general involve both positional and orientational correlations with the specific chemical architecture of the solute, and thus will vary in its details from one molecule to the next. The patchy solute structure generally involves hydrogen bond crisis, since, unlike water, solutes generally will have unequal numbers of hydrogen bond donor and acceptor groups, in addition to the further heterogeneity of polar and non-polar group. A specific reference is made to the content of contributions and abstracts of the Conference Frontiers in Water Biophysics that can be downloaded from the website and from the Special Issue (<http://link.springer.com/journal/11483/6/2/page/1>).

## 2.2. Limonene liquid-vapor partition and partition/coefficients



**Figure 2.4.** Variation of vapour pressure (Pa) of (R)-Limo in water and in 20 % sucrose (SUCR) and 20% trehalose (TRE) as a function of the aroma concentration ( $T= 30^{\circ}\text{C}$ ) ( $CV < 5\%$ )

Release of (R)-Limo in water was determined under thermodynamic equilibrium and in **Figure 2.4**, the variation of the vapor pressure of the aroma as a function of the aroma concentration is reported. The increase of the aroma concentration in the liquid phase determines a linear increase of the limonene content in the gas phase up to concentrations close to those corresponding to its solubility limit in water (13.8 ppm at  $25^{\circ}\text{C}$ , Massaldi and King, 1973) where a step increase of the vapor pressure of the aroma is observed for small higher concentrations of (R)-Limonene. Above 16 ppm, the increasing trend decreases its slope and the system seems to reach a plateau condition.

This liquid-vapor partition behavior could be related to the physicochemical properties of the aroma compound. Limonene is a nonpolar and hydrophobic flavoring agent with



a  $\log P = 4.57$  and limited water solubility and this influences its diffusion and colloidal state in aqueous systems. Above its solubility limit, limonene tends to form a colloidal dispersion in form of micelles or nano-droplets that are less hydrophilic and less soluble. Under this conditions limonene is more prone to reach the water-air interface and to partly in the gas phase. This behavior is also confirmed by the surface tension data reported in Section 2.1. and could be also implied in stripping phenomena during processing (e.g. evaporation, drying) thereby favoring its loss (Soottitantawat et al., 2003).

When a volatile compound reaches its saturation in the condensed phase its concentration does not increase in the vapor phase even if higher concentrations above its solubility limit are considered (Massaldi e King, 1973).

In **Figure 2.4**, the vapor pressure of the aroma added in trehalose and sucrose solutions at 20 % w/v as a function of the limonene concentration in the liquid phase is also reported. The trend is similar at low concentrations of aroma up to 5 ppm and limited and no statistically significant effects were observed due to the presence of the saccharides in the liquid phase in respect to water. Above this concentration an almost linear increasing trend occurred at increasing added limonene in the sugar solution with no meaningful changes in the vapour pressure in correspondence of the concentration corresponding to the water solubility limit. Moreover, a lower ( $p < 0.05$ ) vapor pressure in comparison with that found in water could be evidenced and a significant change between the vapor pressure of limonene in the two saccharide solutions could be observed ( $p < 0.05$ ).

These data could be related to different colloidal properties of the limonene in the TRE and SUCR solutions that may be caused by changes in the solubility of the aroma compound. A solubility of 13.5 mg/L in 20 % sucrose solutions have been reported by Massaldi e King, 1973 while data related to TRE solutions are not yet available. However these results deserve further investigations.

The presence and concentration in the vapor phase of a volatile compound added in a liquid solution depends on several factors. Lower is the volatility, higher is the retention (Bangs and Reineccius, 1982; Savarcos and Moyer, 1968). The vapour

pressure of the pure compound is an index of the volatility of a volatile compound and in the case of limonene it results to be rather low in respect to other more polar compounds (Terta et al., 2006).

The liquid vapor partition of (R)-Limo (2.5 ppm) in water and in TRE and SUCR solutions at different concentrations of the saccharide have been determined and in **Figure 2.5** the retention index, R (%) computed according to the following equation:

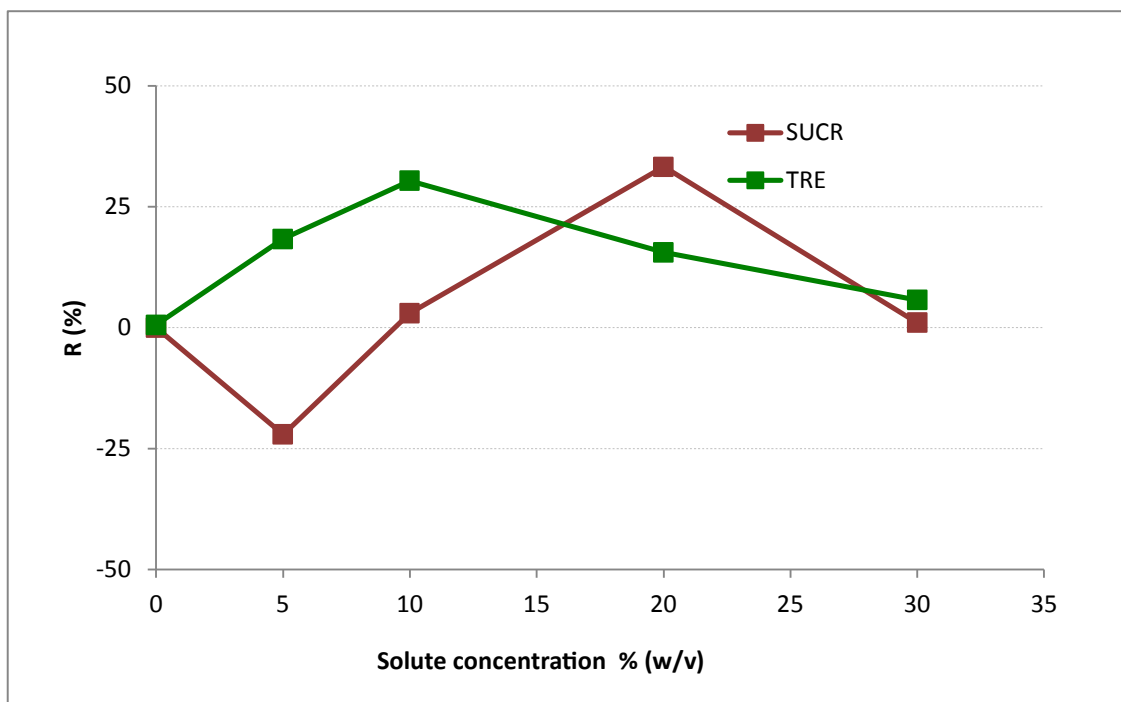
$$R (\%) = (P_w - P_{sz}) / P_w \times 100$$

where  $P_{sz}$  corresponds to the vapor pressure of limonene in the sucrose or trehalose solution and  $P_w$  to the vapor pressure of limonene in water, are reported. The equation has been applied on the vapor pressure data differently to the original study of Seuvre et al., (2006) where it has been used on the liquid-vapor partition coefficients. However, for sake of clarity, positive R values correspond to a retention of the volatile compound whilst negative data are index of a release in respect to the aroma aqueous system.

It could be seen that by changing the saccharide concentration in the liquid phase limonene changes significantly its partitioning in the vapour phase. Composition, chemical and physico-chemical properties of the liquid phase are known to affect the behavior of volatile molecules. Both sucrose and trehalose are influencing differently the release of limonene by either favoring or unfavoring the release from the solution. An effect of the solute concentration could be also observed.

Some studies have shown a lower gas-chromatographic area or vapor pressure of limonene when added in sucrose solutions (Terta et al., 2006, Reineccius 1998). It is known that solutes could determine salting-in (lower release) or salting-out (higher release) on the vapor partition of the volatiles in liquid systems. Effects related to the change of the molar fraction concentration in the condensed phase or due to the water state ("free" water) are the main factors that have been recognized as affecting this behavior (Friel et al., 2000). Vapor pressure of a volatile compound is affected by the activity coefficient of the aroma in the condensed phase. While no data are available about activity coefficient of either limonene or other volatiles in trehalose solutions, Massaldi and King (1973) found a linear decrease of the limonene activity

coefficient in sucrose solutions that may suggest a higher affinity of the volatile to the sugar solution in respect to water.



**Figure 2.5** Retention index,  $R$  (%), of limonene (5 ppm) in trehalose and sucrose solutions at increasing solute concentration.

Our results however, seems to evidence some differences depending on the nature of the saccharide and its concentration that may hypothesize a different affinity of the limonene in the sucrose or trehalose solutions and a different effect of the corresponding physicochemical properties on the liquid-vapor partition of this hydrophobic aroma compound. Changes in the molar fraction of the limonene along with the water activity of the saccharide solutions and of the activity coefficients may occur simultaneously and affecting in an opposite way the release of the limonene and with different entity depending on the solute type and its concentration and this could explain the no linear trend observed in **Figure 2.5**. At increasing solute concentration the molar fraction of the limonene increases in the liquid phase and a decrease of the water activity occurs of different, even if limited, entity for the sucrose and trehalose solutions. Both these factors could cause an increase of the vapor partition (Voilley et

al., 1977; Dalla Rosa et al., 1994; Pittia et al., 1998, 2006). On the other side, the formation of cluster of sucrose moieties with the development of hydrophobic regions at increasing solute concentration in water solution (Robert et al., 1994) may contribute to increase the affinity of the hydrophobic compound in the condensed liquid phase.

System	K (37 °C)		R (%)
	mean	ds	
water	1.25 <sup>d</sup>	0.09	-
20% TRE sol.	1.54 <sup>c</sup>	0.03	-23.1
40% TRE sol.	1.38 <sup>d</sup>	0.11	-10.4
20% SUCR sol.	2.04 <sup>a</sup>	0.28	-63.0
40% SUCR sol.	1.82 <sup>b</sup>	0.23	-45.7

**Table 2.2** Air-water/solution partition coefficient, *K* (mean and deviation standard, *ds*) retention index (*R*, %) of (*R*)-Limonene in water and in sucrose (SUCR) and trehalose (TRE) solutions at 20 and 40 %w/v (*T*= 37°C, (*R*)-Limonene concentration: 5 ppm)

In **Table 2.2.** the partition coefficients of (*R*)-Limonene in water, sucrose (SUCR) and trehalose (TRE) solutions at 20 and 40 %w/v are reported along with the retention index computed according to Seuvre et al., (2006).

The partition coefficient value found is significantly higher than that reported by Savary et al. 2006 that determined it by using the same PRV method but at 30 °C and by using a limonene not as single compound but in a mix of other aroma compounds that may have favored its retention in water, by increasing its solubility.

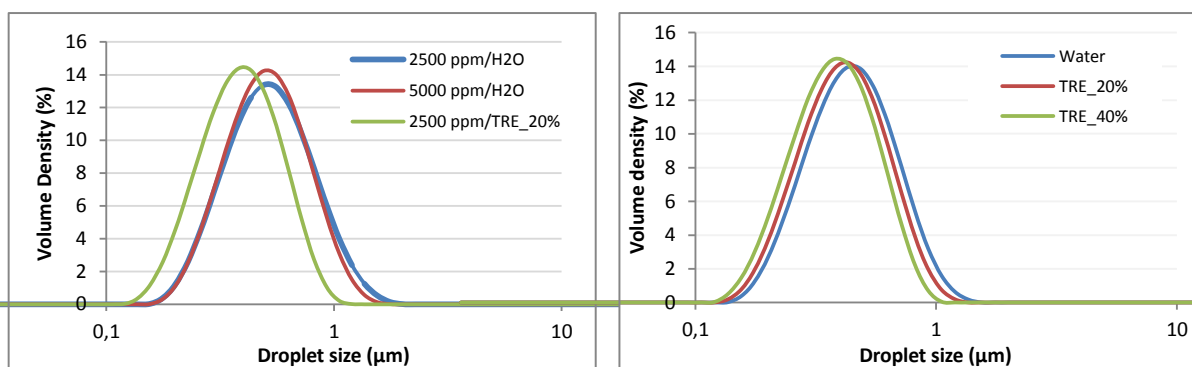
Other data of partition coefficients in water are reported in the literature, both higher and lower than the one found in this study but with different analytical approaches (Head space vs dynamic methods, exponential dilution, temperature of analysis, limonene concentration and time of solubilization of the aroma in water) and methodologies (eg. SPME) (Savary et al., 2006; Nahon et al., 2000; Sadafian and Crouzet, 1987).

Data reported seem however to confirm that, under thermodynamic conditions, an increase of the release of the limonene in saccharide solutions of different entity depends on the nature of the sugar and its concentration. This may be affecting also its retention during storage as well as processing.

### 3. ENCAPSULATING GLASSY MATRICES

#### 3.1. AMORPHIZATION BY CONCENTRATION AND WATER REMOVAL : SPRAY-DRYING AND FREEZE-DRYING

##### 3.1.1. Characterization of the physical and colloidal properties of the initial binary (water /sugar) and ternary (water/sugar/limonene) systems.



**Figure 3.1.1.** Particle size distribution of emulsions made of (R)-LIMO at 2500 ppm (a) and 5000 ppm (b) in water or in trehalose solutions at 20% and 40%.

Particle size analysis of the limonene-in water and limonene-in-TRE solutions at different saccharide concentrations evidenced in all cases a monomodal particle size distribution (**Figure 3.1.1.**) that resulted slightly wider in the case of the simple emulsions made of limonene and water. Independently on the continuous phase composition, the mean droplet size was of sub-micron order and the resulting system could be then defined in the range of nano- and micro-emulsion.

In water, at increasing concentration of limonene, emulsification determined the formation of progressively lower size droplets ( $D_{[4;3]}$  and  $D_{[3;2]}$ ) and corresponding aroma/solution interface (**Table 3.1.1**). This occurred also when limonene was emulsified in the concentrated TRE solution (40%) while no significant difference in the mean diameter of the aroma droplets was observed when dispersed in the 20% TRE solution. In general the presence of TRE provided a LIMO emulsion of smaller mean diameter with an effect that depends on the concentration of the saccharide. This result must be related to the surface tension of the limonene in water and in TRE solutions following also the behavior observed in the experiments (*see Figure 2.3*).

The presence of co-solutes like sugars or alcohols in the aqueous phase seems to favor the adsorption of amphiphilic molecules at the transient air/water and oil/water interface during emulsion formation, with the development of a fine emulsified system with droplet of small size. The positive contribution of sugars in the emulsification process as well as in improved stability of the emulsions have been recently observed also by other authors (Maher et al., 2014; Sosa et al., 2014; Ikeda et al., 2013; Huck-Iriart et al., 2013).

Aqueous phase composition	(R) - Limo concentration (ppm)	D <sub>[4;3]</sub> (µm)		D <sub>[3;2]</sub> (µm)	
		Mean	SD	Mean	SD
H <sub>2</sub> O	2500	0.559 <sup>a</sup>	0.030	0.472 <sup>a</sup>	0.024
	5000	0.461 <sup>bc</sup>	0.017	0.394 <sup>c</sup>	0.013
20 %w/v TRE sol.	2500	0.507 <sup>b</sup>	0.018	0.435 <sup>b</sup>	0.025
	5000	0.443 <sup>c</sup>	0.017	0.379 <sup>c</sup>	0.014
40%w/v TRE sol.	2500	0.470 <sup>b</sup>	0.015	0.408 <sup>c</sup>	0.012
	5000	0.409 <sup>d</sup>	0.014	0.352 <sup>d</sup>	0.012

SD: standard deviation

**Table 3.1.1** Volume-weighted mean diameter  $D_{[4;3]}$  (µm) and volume-surface mean diameter  $D_{[3;2]}$  (µm) of the emulsions obtained by emulsification of (R)-Limo (2500 and 5000 ppm) in water or in TRE solutions at 20% and 40% of solute concentration.

Changes in the dispersion degree of limonene microemulsions were determined in order to optimize the following process drying conditions and timings.

Limo-water microemulsions upon 24 h of storage conditions at room temperature showed some flocculation and coalescence phenomena with the appearance of a bimodal particle size distribution at higher particle size. For the 2500 ppm systems the  $D_{[4;3]}$  was equal to  $2.34 \pm 0.08$  µm and the  $D_{[2;3]}$  to  $1.07 \pm 0.04$  µm. On the contrary, in the TRE-based emulsions, a monomodal distribution of the limonene dispersed droplets was still observed. A limited, but statistically significant, increase of the  $D_{[4;3]}$  and  $D_{[3;2]}$  in respect to the initial values (0.80-0.85 µm and of 0.59-0.62 µm, respectively) was observed but without effects due to the saccharide concentration in the aqueous phase.

### 3.1.2 Characteristics of dried, amorphous powders and limonene retention

#### 3.1.2.a Processing dehydration conditions: spray-drying (SD) vs freeze-drying (FD)

Spray drying is a technology widely used in large-scale production of encapsulated flavors and volatiles (Uhleman and Reiss, 2010) as well as other liable compounds due to its short-time process, with minimal thermal damage to the final product. This convective drying technique transforms the initial dispersion of atomized droplets into a dry, free-flowing powder.

Some organic molecules and, in particular, low molecular weight carbohydrates are used in spray drying to protect labile molecules, such as proteins, from conformational changes and chemical degradation that may originate during drying process and/or due to storage stressing factors (e.g., humidity, temperature). Previous investigations have been carried out on spray dried formulations made of trehalose to evaluate the physical properties of the low-moisture, glassy systems in absence/presence of other co-solutes (Sussich & Cesàro, 2008; Moran & Buckton, 2007). Freeze-drying is a mild drying technology where water loss is induced by sublimation from matrices in frozen state and at low pressures. While process conditions limit the degradation of the most liable compounds and the loss of the most volatile compounds, it is used for flavor encapsulation only for highly valuable products and to a limited extent due to its high costs.

In **Figure 3.1.2**, thermograms of spray-dried (S-D) systems produced from trehalose solution (20%) and trehalose+ R-Limo microemulsion (20 % trehalose, 2500 ppm limo) are shown. In our laboratory few experiments have been also carried out to obtain low moisture glassy systems by freeze-drying on trehalose aqueous systems with or without (R)-Limo in order to investigate the effect of reduction of water from a different physical state of water and the corresponding effects of limonene retention. Thermal properties of the corresponding samples obtained from the same trehalose aqueous systems (F-D) with or without limonene have been investigated and thermograms are also reported in **Figure 3.1.2**.

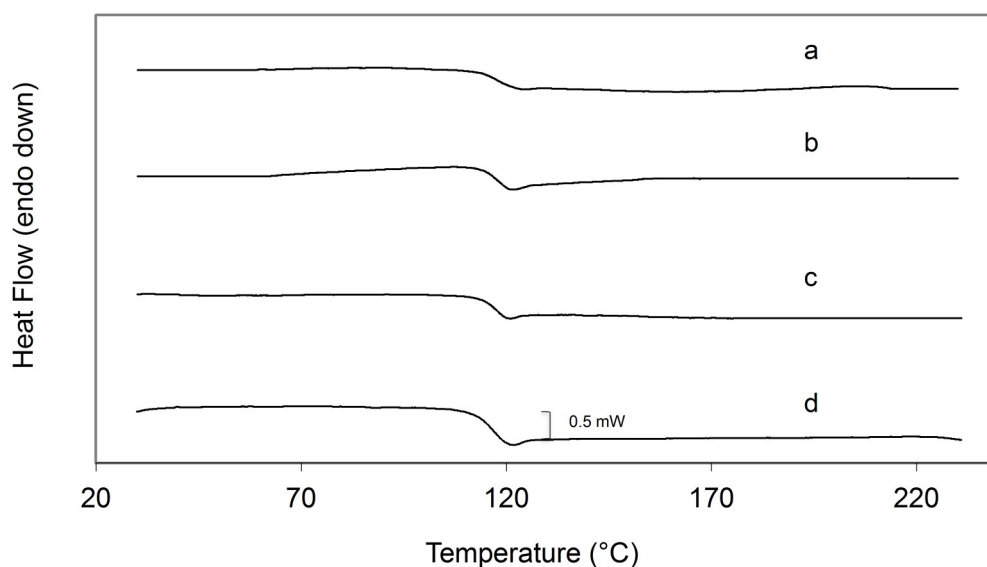
The low moisture powders, independently on the presence of limonene and on the technology used for moisture removal, showed a similar trend upon heating. In particular the systems present a baseline shift at temperatures between 115 and 120 °C that was attributed to the trehalose glass transition, above which they appear unable to undergo cold crystallization. Similar behavior has been observed by Sussich and Cesàro (2008) in amorphous trehalose obtained by both freeze-drying and spray-drying even if at different process conditions as well as by Willart and Descamps (2007) by quenching liquid trehalose. No other specific analysis has been carried out to assess the characteristics of the powder that, however, it is shown to be amorphous by the presence of the glass transition.

In samples of dried trehalose, an enthalpy relaxation endotherm or a T<sub>g</sub> overshoot is usually observed, as well as in the freeze-dried samples made with limonene, while this endotherm is not present in the corresponding spray-dried ones. Enthalpy relaxation is a signature of the storage of a glassy state at a temperature sufficiently close to T<sub>g</sub> for a sufficient time to allow aging in the glassy matrix. Therefore the interplay of time and temperature is necessary. Since the temperature of the limonene-containing glassy trehalose is not affected by the limonene presence (absence of plasticization effect), it seems evident that the presence of the endotherm is solely due to a combination of the presence of moisture (decrease of T<sub>g</sub>) and process specification (temperature and time of annealing). However, a detailed analysis of this phenomenon has already been given in literature for some circumstances and is outside of the scope of the present work.

The presence of limonene in both F-D and S-D powders did not affect the glass transition as samples analyzed just after drying presented a T<sub>g</sub> of ca 115°C independently from the drying process and presence of limonene. This could be related to a residual amount of water, since the original moisture of the just made samples was 6.4 +/- 0.1 % and 9.76 +/- 0.23 for the SD trehalose and limonene-trehalose powders, respectively.

After storage under P<sub>2</sub>O<sub>5</sub> the T<sub>g</sub> of the same powders increased to values close to that of anhydrous trehalose (ca. 119°C).





**Figure 3.1.2:** Thermograms of spray-dried (S-D) (a, b) and freeze-dried (F-D-) (c, d), starting from 20 % TRE solution with (R)-Limo (2500 ppm, a, c) or without (b, d). SD process conditions:  $T_{inlet}$  120°C. FD: see M&M section.

Process	Sample	$T_{g_{mid}}$ (°C)	Notes
Spray-drying (S-D)	TRE ( $a_w=0.11$ )	$115.5 \pm 0.3^b$	No cristallization
	TRE +(R)_Limo ( $a_w=0.11$ )	$115.4 \pm 0.4^b$	Some small exothermic peaks ( $T > 146^\circ\text{C}$ )
	TRE ( $a_w < 0.05$ )	$119.5 \pm 1.0^a$	No cristallization
Freeze-drying (F-D)	TRE ( $a_w=0.11$ )	$116.3 \pm 1.6^b$	No cristallization
	TRE +(R)_Limo ( $a_w=0.11$ )	$115.4 \pm 0.4^b$	No cristallization
	TRE ( $a_w < 0.05$ )	$119.2 \pm 1.6^a$	No cristallization

**Table 3.1.2:** Glass transition temperature of spray-dried and freeze-dried TRE and TRE+(R)\_Limo

	<b>(R)-Limonene retention (%, on the initial)</b>	<b>retention- SURFACE %</b>	<b>retention – ENCAPSULATED (%)</b>
F-D	25.0 ± 1.3 <sup>b</sup>	5.4	94.5
S-D	31.3 ± 1.2 <sup>a</sup>	1.5	98.5

**Table 3.1.3:** Limonene retention (% total, surface and encapsulated in respect to total) in freeze-dried (F-D) and spray-dried (S-D) powders obtained from an initial limonene (5000 ppm) emulsion in TRE solution (20 %w/v).

In **Table 3.1.2** the results of the limonene retention total, superficial and encapsulated (the latter expressed as % on the total of the retained limonene) of the F-D and S-D trehalose powders are reported. Under the experimental conditions used in this study, a higher retention of limonene in SD samples (31%) has been observed with a relative higher encapsulation ratio in comparison with the freeze-dried ones (ca. 25%). In general, it is expected that the retention of volatile flavor compounds during drying is higher when lower temperature conditions are applied. We would expect that part of the limonene is lost after the freeze-drying, since the grinding step carried out to obtain the final powder bring limonene to the surface fractures. This hypothesis could be demonstrated by the higher presence of limonene on the surface of the FD powders (ca 5%) compared to that determined in the SD ones (ca. 1.5%), taking into account also that SD powder was not subjected to this action. If no specific interactions occur between limonene and amorphous trehalose on the surface, it could easily diffuse in the gas phase surrounding the sample. Mention to possible hydrophobic interaction with sugars is necessary in view of the current findings of apolar interactions of sugars with partially hydrophobic molecules, as caffeine (Tavagnacco et al. 2012).

No data in the literature are available on the retention of limonene in both spray-dried and freeze-dried made starting only from small carbohydrate matrices and trehalose in particular. Some data on the retention of volatile polar compounds (n-alcohol series, from methanol to 1-pentanol) in F-D mon- and di-saccharides based matrices (glucose, sucrose, lactose, maltose, initial solution concentration: 20% w/w) are reported in Flink

and Karel (1970). In their study, the highest retention was observed in F-D sucrose for volatiles with low vapor pressure and a relative low polarity, from 50 to 75%.

It is known that several factors influence the retention of the volatiles by affecting in turn the physical and structural properties of the dried, low moisture products. The nature of these factors can be both intrinsic (e.g. type of solute and its concentration, aroma compound nature, properties and concentration) or extrinsic which mostly include process conditions such as pressure, freezing rate, temperature profile during drying for FD and inlet temperature, pressure and atomization conditions for SD.

It is known that main factors that affect the encapsulation mechanisms and the retention during spray-drying. In particular, hydrophobicity strongly affects the extent of phase separation of the aroma compound into discrete droplets versus their “dissolution” state in the carrier, while the vapor pressure influences mainly the retention of the flavor. The detailed analysis of the influence of these factors was in part out of the scope of this research that was mostly aimed at better understanding the role of the presence of an apolar volatile compound (i.e. limonene) on the physical, thermal and structural properties of the low moisture, glassy saccharide-made matrices. However, based on these preliminary results, some investigations to characterize the effect of process conditions during spray drying and, in particular, the inlet temperature along with the composition (i.e. concentrations of limonene and trehalose in the starting emulsion) were carried out.

### **3.1.2.b Effect of SD process conditions ( $T_{inlet}$ , [TRE], [Limo])**

The effect of some SD process conditions, and, in particular, the inlet temperature at the atomizer level (from 110°C to 160°C), trehalose concentration (20 and 40 % w/v) and limonene (0-10000 ppm) was investigated to better define their role on the physical and structural properties of the corresponding amorphous powders as well as the retention of encapsulated limonene. It has to be pointed out that this series of experiments was carried out in a time following the very preliminary ones and discussed above in Section **3.1.2.a**.

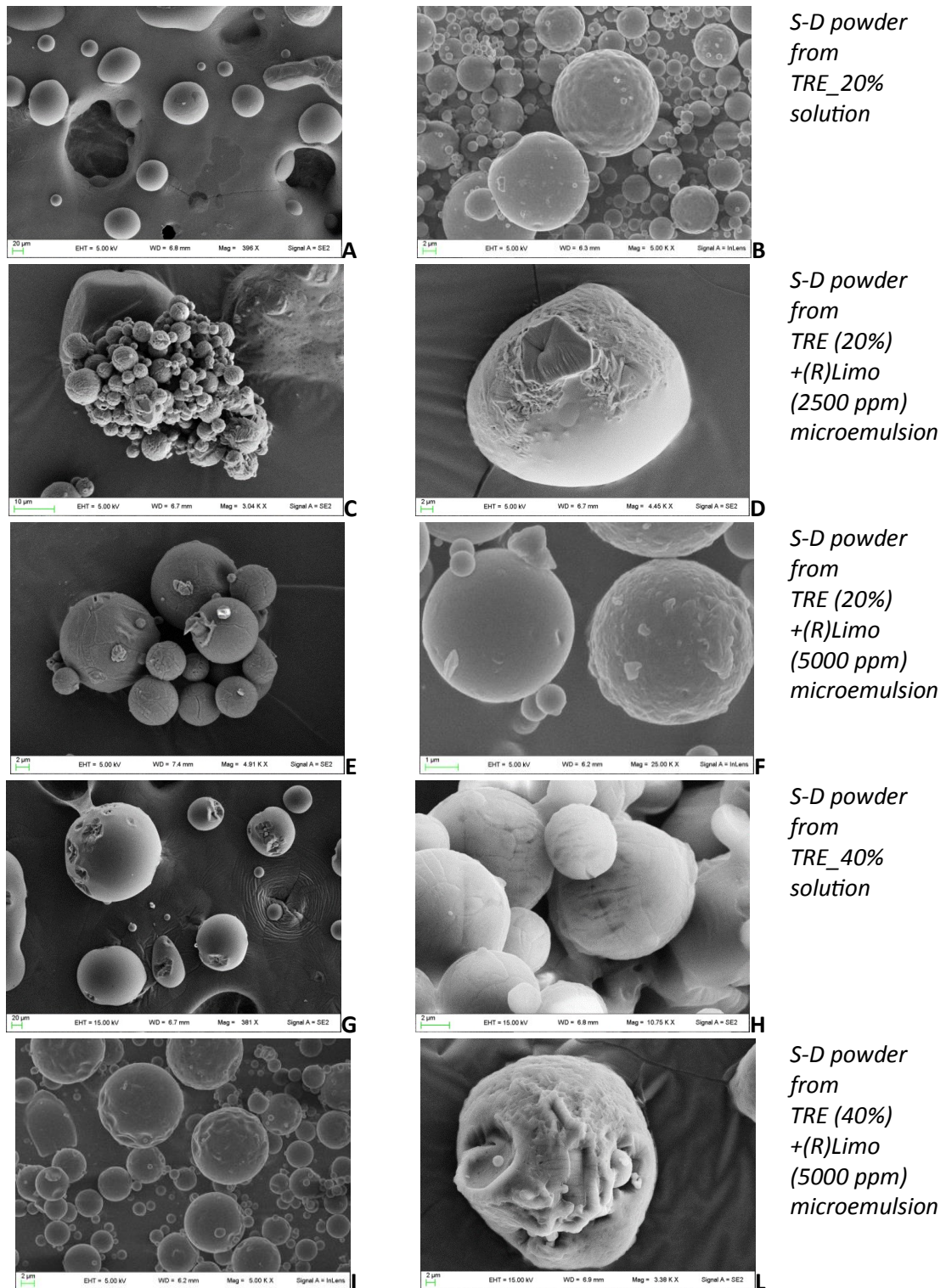
Unfortunately, the spray-drier instrumentation previously used was subjected to major equipment maintenance services that, however, compromised its full drying efficiency under the same process conditions (biased vacuum control). This issue resulted clearly by comparing the moisture content and the limonene retention of the powders obtained from the same initial trehalose-limonene liquid system (see results below). To strengthen the significance of the results and the process/formulation effects, results reported and discussed in this session correspond to the analyses carried out on samples obtained from at least two repetitions of spray-drying experiments carried out in different times and thus from different initial starting liquid systems. Microstructural and thermal investigations out along with the retention of limonene in the dried powders were carried.

Infeed system composition			Moisture (%)	
$T_{inlet}$ (°C)	TRE (% w/v)	(R)-Limo (ppm)	mean	SD
120	20	0	12.6	3.0
120	20	2500	12.9	3.0
120	20	5000	11.5	2.2
120	40	5000	11.6	2.2
120	40	5000	12.9	3.8
110	20	2500	12.9	4.0
110	20	10000	8.1	2.5
160	20	10000	9.2	3.4
160	20	2500	10.1	2.8
145	20	6250	10.9	3.5

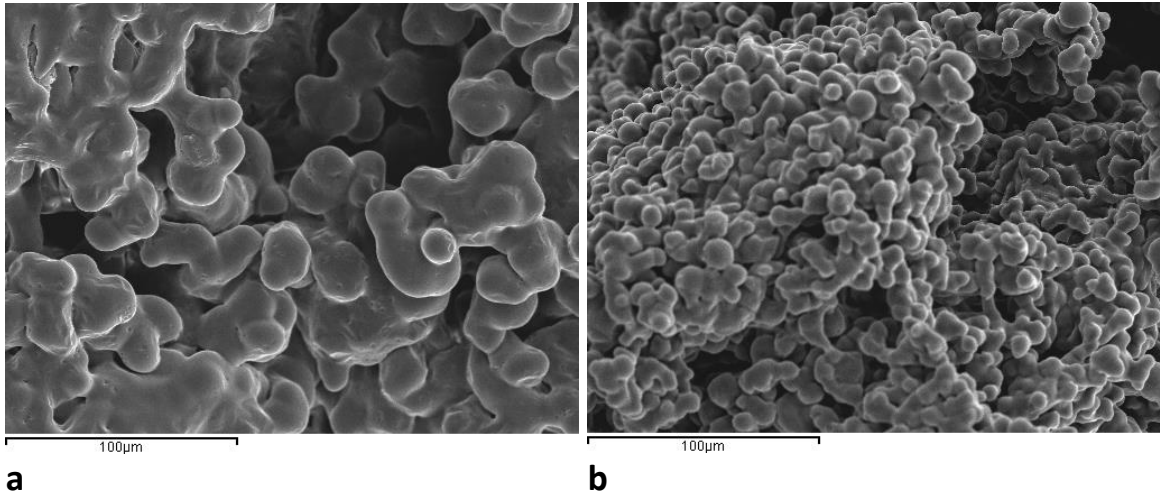
**Table 3.1.3.:** Moisture content of SD powders obtained from differently formulated and processed TRE solutions and limonene-trehalose microemulsions.

In **Table 3.1.3**, moisture of the amorphous powders obtained from differently formulated and processed TRE solutions and Limo-TRE microemulsions. A high moisture content (> 8 %), higher than that found in the preliminary experiments was determined, with lower values from the powders made from Limo-TRE microemulsions and the higher ones for those prepared with no aroma in the initial system. The

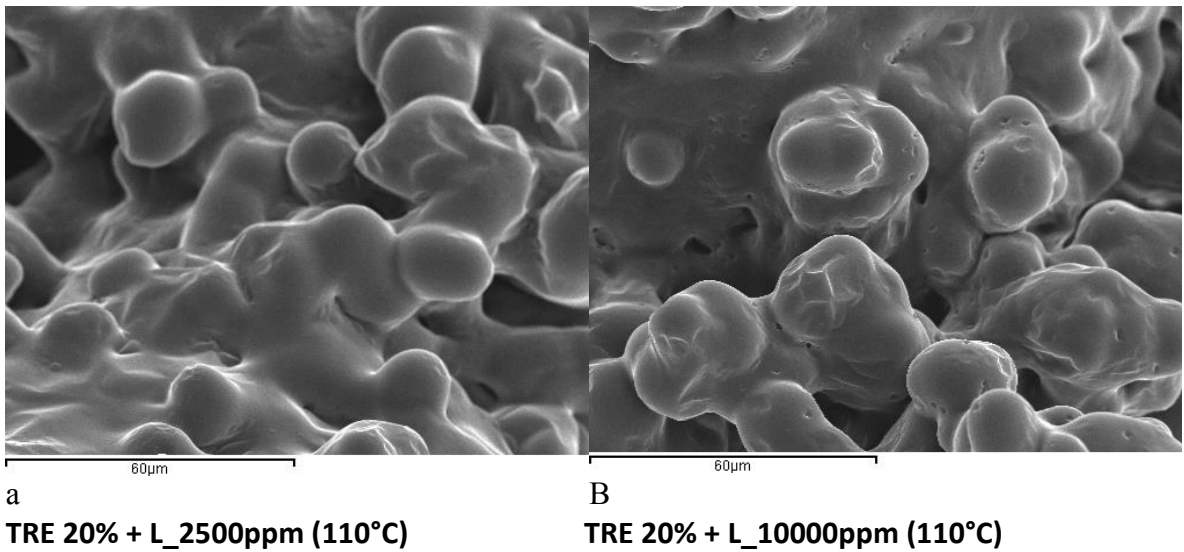
presence of the volatile compound the “simple” aroma-saccharide dispersion and the absence of any additional film forming compound or emulsifier could have somehow favoured the water removal from during the process as the lower moisture is shown in the samples prepared with the higher content of limonene in the feeding system. Several S-D experiments have been carried out with an inlet temperature of the atomizer at 120°C in order to investigate the effect of the initial solution composition. Scanning electron microscopy images of the S-D powders of these samples are shown **Figure 3.1.3.**, and the mean diameter size of the particles are reported in **Table 3.1.4.** The images have been collected and reported at different magnifications in order to highlight the specific microstructural features. Under these process conditions, the particles produced by spray-drying TRE solutions at 20% solute concentration (Figure Fig.3.1.3. a, b) show small particles with a spherical shape that appears less regular than those obtained from the solution at 40% concentration (Fig.3.1.3. g, h) with higher dimensions (Table 3.1.4.). Similar results were observed by Moran and Bukton (2007) and Adler and Lee (1999) by spray-drying trehalose solutions from 0.5 up to 10% of the disaccharide and typical also of spray-dried sugars such as lactose and mannitol. Independently from the initial solution concentration, all powders generally showed a smooth and regular surface; however, the powder obtained from the 20% trehalose solution show partly agglomerated and fused particles, while those from the 40% trehalose solution show some surface defects. The current interpretation of size development during spray-drying is that dimensions and shape of the particles are determined by the step during droplet evaporation when the solid matrix starts forming. This event must result at a given stage of the evaporation process occurring in the atomizer; the droplets with low solute content are formed at a later stage, because of a longer process of evaporation prior to the sugar precipitation onset. Longer time of evaporation may cause not regular shapes and favor particle aggregation and coalescence.



**Figure 3.1.3:** Microstructure of spray-dried (S-D) trehalose and trehalose + limonene systems obtained from initial systems at different saccharide and limonene concentration ( $T_{inlet}$ : 120°C).



**Figure 3.1.4:** Microstructure of spray-dried trehalose + limonene powders obtained from initial liquid systems made of 20 % trehalose and 10000 ppm (R)-Limonene microemulsion dried at two different inlet temperature: 110°C (a), 160 °C (b).



**Figure 3.1.5:** Microstructure of spray-dried trehalose + limonene powders obtained and from initial liquid systems made of 20 % trehalose and 2500 ppm (a) and 10000 ppm (b) limonene as microemulsion by applying an inlet temperature of 110 °C.

S-D process condition	Initial composition of the liquid system		Ferret diameter ( $\mu\text{m}$ )	
	$T_{\text{inlet}}$ ( $^{\circ}\text{C}$ )	TRE (% w/v)	[R-Limo] (ppm)	Mean
120	20	0	39	22
120	40	0	50	28
120	20	2500	18	11
120	20	5000	8	4
<b>120</b>	<b>40</b>	<b>5000</b>	33	12
110	20	2500	20	9
110	20	10000	10	5
160	20	2500	11	3
160	20	10000	8	3

**Table 3.1.4.:** Mean particle size of spray dried powders obtained from trehalose solutions at different solute concentrations and limonene emulsions in trehalose solutions at different composition and by applying different inlet temperatures.

When the limonene-trehalose microemulsion was used, relevant differences were observed in both morphology and size of the powder particles. In general, the presence of limonene determined the formation of dried particles of round and regular shape, with a significantly smaller size compared to those obtained from the corresponding trehalose solution without the volatile. Changes in the limonene concentration in the feeding liquid system affect the particle size. The dried particles encapsulating limonene present a rough surface and clearly visible pores and breaks on the surface as a consequence of the residual evaporation of water and limonene in the final step of drying (El Sayed et al., 1990, Reineccius, 2004).

Interestingly enough, the formation of these surface holes is a phenomenon commonly observed on evaporation of water from a polymer solution film and has been attributed to a characteristic unbalance between the rate of water and the rate of polymer diffusion in the concentrated solution. In the powders obtained from emulsion with high limonene and trehalose concentration an irregular surface with wrinkles reflecting a very pronounced shrinkage is observed. Shrinkage is a phenomenon known to occur in the final step of the drying within the spray-drier



chamber just before the particles reach the solidified morphology (El Sayed et al., 1990).

At high temperature (160 °C) and similar initial composition of the feeding liquid system, powder particles with a low mean size are produced (Figure 3.1.4., **Table 3.1.4**); this is in agreement with the higher evaporation rate during the process (driving force), while no difference apparently occurs between the particle size of the samples dried at 110 °C and 120 °C.

At low temperature (110 °C) the presence of emulsified limonene at high concentration in the initial feeding liquid system caused an increase of the shrinkage effect that could only partly be evidenced from the micrographs reported in **Figure 3.1.5.**, because of the agglomeration induced by the residual moisture not immediately removed after spray-drying and prior to SEM analysis.

In **Table 3.1.5.**, the retention of limonene in the trehalose powders (expressed in % in respect to that ideally present in the powder) made from solutions with different concentration ratio of carrier/limonene and subjected to different drying temperature is summarized. In general, limonene resulted almost completely encapsulated in the trehalose low moisture powder particles as % of the limonene determined at the surface of the powder particles was in all samples lower than 0.2%. It can be noticed that when the feeding solution had an initial concentration of 20%, the limonene retention in the carbohydrate amorphous powder was in the range of 10 to 19 % with a scarce effect of the drying temperature as well as of the initial limonene concentration, despite the fact that significantly different concentrations of limonene emulsified in the TRE solution (2500 vs 10000 ppm) was tested.

The mechanism of volatile loss during spray-drying has been the subject of substantial research reviewed by Coumans et al. (1994), King (1995) and Reineccius (2004).

Three main steps during drying by atomization have been identified by King (1994) where volatile losses can occur: atomization (pre-droplet formation), after droplet formation but before particle temperature reaches the boiling point of water, and finally, when the particle temperature reaches the boiling point of water but before the final setting of the particle structure.

Feeding systems					
T <sub>inlet</sub> (°C)	Trehalose (% w/v)	Limonene (ppm)	Limonene retention in the dried powder (%)		
			mean	ds	
120	20	2500	19.3 <sup>b</sup>	2.6	
120	20	5000	11.6 <sup>c</sup>	3.8	
120	40	5000	28.9 <sup>a</sup>	5.0	
110	20	2500	12.1 <sup>c</sup>	0.9	
110	20	10000	10.1 <sup>c</sup>	1.7	
160	20	10000	13.1 <sup>c</sup>	2.2	
160	20	2500	11.0 <sup>c</sup>	1.4	
145	20	6250	14.4 <sup>c</sup>	1.5	

**Table 3.1.5:** *Limonene retention in S-D trehalose powders obtained by different process conditions and from initial liquid systems of different composition.*

The main factors contributing to the retention of volatiles during drying by atomization are the rate of formation of a dried, impermeable membrane, which is a physical barrier towards volatile loss, and the selective diffusion mechanisms of the components. The driving force for the mechanism implies that the diffusion coefficient of water declines less than that of the other components as the water concentration decreases in aqueous solutions when the surface reaches a moisture content ranging from 7 to 23%. This phenomenon occurs for carbohydrate-based materials, as well as other substances and it results from the small size of the water molecule, which enables it to move more facily within a highly hydrogen-bonded matrix than the larger and more hydrophobic volatiles.

As no major effects on the retention of the limonene have been observed as a function of its concentration, it is likely that in the presence of trehalose alone in the feeding liquid system, the main loss of this apolar, volatile compound has occurred in the first part of the drying, well before the formation of solid droplet, likely made easy also by its surface properties (see Figure 2.3).

The presence additional components (e.g. surfactants, weighing agents, hydrocolloids, or additional lipids) in the infeed system favor the formation of the droplet by minimizing oscillations, turbulence and internal circulation in drops, and provides a rapid drying of the surface (Frey and King, 1986).

Therefore, the addition of complex carbohydrates (eg. maltodextrins, starches,  $\beta$ -cyclodextrins), hydrocolloids (e.g. gum Arabic) as well as emulsifiers (Tween 20, whey proteins) as well as of small saccharides like sucrose or trehalose, used as weighting agents and in low concentration that those used in this study, could improve the limonene retention in spray-dried systems up to 100 % as shown by several studies (Lui et al., 2001; Bertolini et al., 2001; Roos, Jafari et al., 2007; Yamamoto et al., 2012).

Investigations on retention of spray dried hydrophobic flavors and/or essential oils evidenced in general that a higher retention of volatiles is achieved by decreasing the emulsion size (Soottitantawat et al. 2003, 2005; Risch and Reineccius, 1988; Re´ and Liu, 1996). However, this condition does not result in a higher stability after processing and shelf life of the encapsulated compounds due to the higher surface exposed to external stresses (Risch and Reineccius, 1988). Furthermore, in maltodextrins based systems added with Tween 20 as emulsifying agents, the formation of a limonene emulsion (20%) with droplets at nanoscale size ( $D_{[4.3]} < 200$  nm) resulted in a powder with a relatively poor encapsulation efficiency (about 75%).

At the same limonene concentration (2.5%), higher retention of limonene in the powder was obtained at higher infeed trehalose concentration (Table 3.1.5). This is in agreement with the retention mechanism upon drying and with the large literature studies aimed at exploring the effect of infeed solid content, as reported in Reineccius (2004). High infeed solids increase retention during drying, primarily by reducing the time needed for the formation of a semipermeable membrane at the drying particle surface and by hastening the onset of selective diffusion of water and volatiles. An optimum solute/carrier concentration of the infeed system is generally defined for flavor retention primarily based on solubility and viscosity in solution (Reineccius, 2004).

**Figures 3.1.6.** and **3.1.7.** show thermograms of the differently processed TRE powders and TRE+(R)-Limo encapsulates obtained from differently formulated infeed systems. They are representative of the thermal behavior observed in the several repetitions of the spray-drying experiments. The corresponding thermal data are reported in **Table 3.1.6.**

Spray drying in presence of sugars is widely applied to protect biomolecules (such as proteins) against conformational changes and chemical degradation triggered by the drying conditions as well as by environmental stress agents during storage (e.g. Sussich and Cesàro, 2008, Drusch et al., 2006).

Previous studies on the use of trehalose in spray-died formulations indicate the possibility to obtain powders in a fully glassy state when referred to the systems made of trehalose alone. This was confirmed by wide-angle-X-ray scattering diffraction analysis showing the characteristics of amorphous halo (Adler and Lee, 1999). Thermograms obtained from the undercooled liquid of melt crystalline trehalose-  $\beta$  in general present only a heat capacity step at the glass transition temperature; further heating above  $T_g$  do not show other phase transitions such as cold crystallization and melting (Sussich and Cesàro, 2008).

In our samples the differently formulated trehalose powders with or without encapsulated limonene showed a thermal behavior similar to that of the amorphous powders with a  $T_g$  comprised between 116°C and 118°C, only slightly lower than the anhydrous trehalose glass. This small difference is ascribed to traces of residual water not removed after processing (Crowe et al., 1996). Although no evidence of cold crystallization could be observed, endothermic peaks that could be referred to the melting of crystalline trehalose are observed at temperatures above 200°C. The enthalpy of these peaks results higher in powders obtained at lower  $T_{inlet}$  (110°C) as well as in those from infeed solutions at high solute concentration (40%). Moreover, based on the shape of the endothermal peak different polymorphic forms of crystals were observed, and in most of the cases they correspond to the melting of trehalose di-hydrate.

These results could be related to the different cooling/quenching rate during drying of the trehalose particles in the drying chamber. Furthermore, the residual moisture in the dried powders could have also promoted the crystallization of the amorphous disaccharide upon equilibration after processing. Trehalose powders could present different physical properties depending on the solute concentration being spray-dried due to their effects on the packing structure (Sussich and Cesàro, 2008).

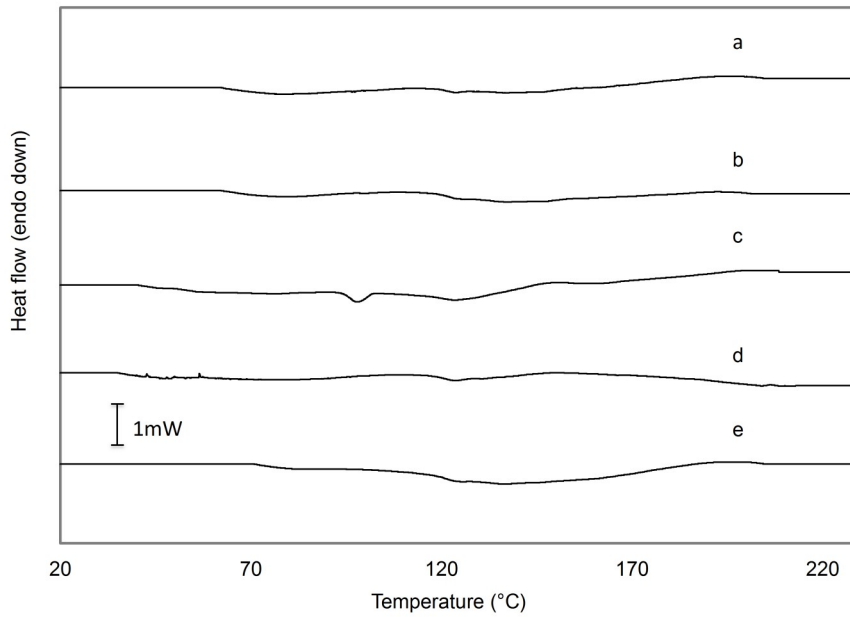
Due to the residual moisture and its recognized effects on both glass transition and crystallization difficult is to evidence a significant role of the encapsulated and/or dispersed limonene on the thermal properties of the amorphous trehalose. The only main effect that could be evidenced in samples dried at 120°C is the lower enthalpy of the melting of the crystalline fraction in the samples related to a higher encapsulated limonene even if the high variability of the thermal data do not allow to evidence a statistical difference.

At increasing limonene concentration in the infeed system, a lower particle power size was obtained and, as a consequence, the more rapid and easy water removal during drying could have reduced the formation of crystalline trehalose in the amorphous matrix.

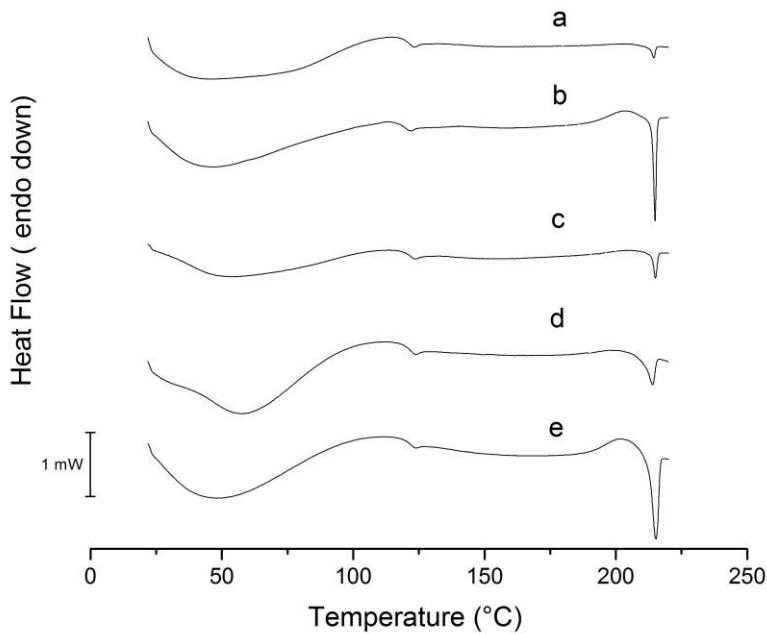
T <sub>inlet</sub> (°C)	TRE (% w/v)	[LIMO] (ppm)	T <sub>g, mid</sub> (°C)		ΔH relaxation at T <sub>g</sub> (J g <sup>-1</sup> )		Endothermic peak (crystals melting)					
			mean	DS	mean	DS	T <sub>onset</sub>		T <sub>peak</sub>		ΔH (J g <sup>-1</sup> )	
							mean	DS	mean	DS	mean	DS
120	20	0	116.5	0.95	0.29	0.06	208.6	3.0	209.7	4.0	2.3 <sup>c</sup>	1.4
	20	2500	117.4	0.48	0.36	0.06	207.8	3.4	210.0	3.1	2.3 <sup>c</sup>	1.2
	20	5000	116.7	1.36	0.35	0.04	208.4	1.3	209.3	0.9	1.7 <sup>c</sup>	0.8
	40	0	116.6	0.54	0.23	0.05	209.5	0.8	211.7	0.2	9.9 <sup>ab</sup>	1.6
	40	5000	117.0	1.04	0.42	0.11	206.8	3.8	210.1	4.3	4.9 <sup>b</sup>	3.5
110	20	2500	117.4	0.56	0.34	0.05	212.4	2.7	213.4	2.1	15.3 <sup>a</sup>	6.2
110	20	10000	118.7	0.21	0.32	0.08	212.3	1.7	213.6	1.9	1.7 <sup>c</sup>	0.7
160	20	10000	118.9	0.32	0.39	0.07	209.6	2.6	212.5	1.6	3.9 <sup>c</sup>	1.2
160	20	2500	119.2	0.37	0.32	0.04	211.3	2.8	213.4	2.0	5.9 <sup>b</sup>	1.8
145	20	6250	118.8	0.38	0.33	0.03	212.3	3.7	213.5	1.6	1.1 <sup>d</sup>	0.2

T<sub>g, mid</sub>: T<sub>g</sub> at mid ΔC<sub>p</sub>; T<sub>c, onset</sub>: initial temperature of re-crystallisation; T<sub>c, peak</sub>: max temperature of the crystallization peak; ΔH<sub>c</sub>: enthalpy of crystallization; T<sub>m, onset</sub>: initial temperature of melting; T<sub>m, peak</sub>: max temperature of the melting peak; ΔH<sub>m</sub>: enthalpy of melting. <sup>a-f</sup>: different letters means significant difference (p<0.05) of the means in the same column

**Table 3.1.6:** Summary of transition temperatures and thermal data of the S-D TRE and TRE\_(R)-Limo powders obtained by different drying conditions and from initial systems of different solute and limonene concentration.



**Figure 3.1.6.** Thermograms of S-D powders made from 20 % (a) and 40 % (b) TRE solutions and TRE-(R)-Limo microemulsions made with the following composition: 20 % TRE-2500 ppm Limo (c), 20 % TRE-5000 ppm Limo (d); 40 % TRE-5000 ppm Limo (e).



**Figure 3.1.7.** Thermograms of S-D TRE-20 % solution (a) and of TRE\_(R)Limo microemulsions of different composition and dried under different process conditions: TRE (20%)\_ Limo-2500 ppm ( $T_{inlet}$ : 110°C) (b); TRE (20%)\_ Limo-10000 ppm ( $T_{inlet}$ : 110°C) (c); TRE (20%)\_ Limo-10000 ppm ( $T_{inlet}$ : 160°C) (d); TRE (40%)\_ Limo-5000 ppm ( $T_{inlet}$ : 120°C).

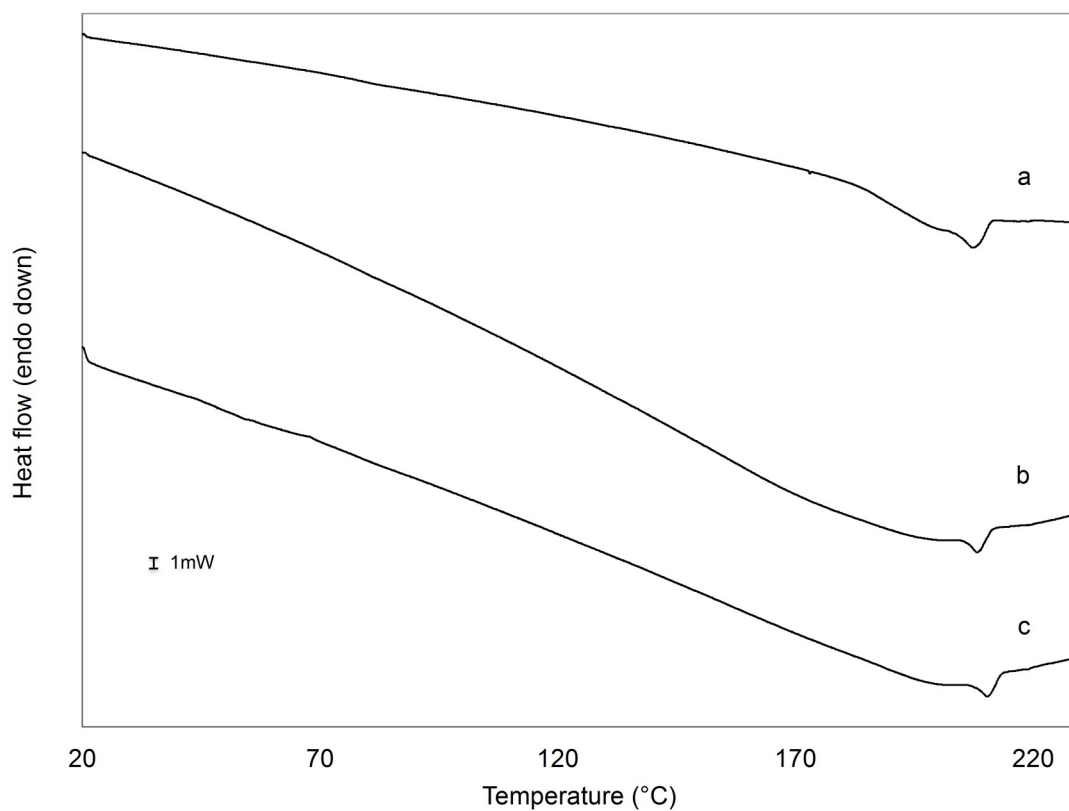
NMR analysis have been carried out to investigate the dynamic properties of the spray-dried TRE and TRE+Limo powders obtained from initial systems of different formulation and subjected to different process conditions. The aim is to complete the study of the relations of the entrapped aroma and the physical state of the systems. The relaxation time of a complex population decreases with the increasing mobility of the components. Under the elaboration approach used in our study, two relaxation times were considered: the “average” relaxation time ( $a-T_2^*$ ) and “total” relaxation time ( $t-T_2^*$ ), where the latter is usually associated only to the liquid component of the system. In our samples the  $a-T_2^*$  value of the dried systems resulted statistically correlated with the residual content of limonene ( $R>0.98$ ), with a lower  $a-T_2^*$  value in systems with lower encapsulated limonene. This results in agreement with the findings of Andrade et al. (2008) that found a similar result by using low field NMR to determine the content of limonene encapsulated in maltodextrin and arabic gum spray-dried powders. This result reflects the fact that in the encapsulated powders the hydrophobic limonene exists as discrete liquid “oil” droplets with little interaction with the carrier (Andrade et al., 2008).

The second moment,  $M_2$  reflects the strength of dipolar interactions in which rigid protons are involved (van Den Dries et al., 1998). Results of the NMR analysis on the initial crystalline TRE-b and the differently prepared spray dried samples, a significant decrease of the  $M_2$  values was observed. In the former average values of  $9370 \text{ s}^{-2}$  were determined while in the latter the data were in the range between 7700 and  $7850 \text{ s}^{-2}$ . No significant differences among the samples made starting from TRE solutions and limonene dispersions at 20 % solute concentration that resulted significantly higher than those observed when TRE solutions at 40% were dried. The decrease of the second moment  $M_2$  value from the crystalline state and the amorphous one results thus significant and reflecting the main physical change occurred in the matrix due to solvation and desolvation processes. The concentration of the initial feeding systems resulted also a significant factor in decreasing the strength of the dipolar interactions associated with an increased mobility of the rigid protons in the matrix. The decrease

of this parameter, however could be also associated to the high water content (Roudaut et al., 2009).

### 3.2. MILLING AND CO-MILLING

#### 3.2.1. Thermal properties of mix of crystalline TRE and (R)-Limo



**Figure 3.2.1:** Thermograms of  $\beta$ crystalline trehalose added with different (R)-Limo concentrations. a) pure crystalline TRE -  $\beta$ ; b) TRE -  $\beta$ + 2.7%w/w Limo; c) TRE -  $\beta$ + 11.0 % w/w Limo

In order to better understand and to describe the effects of the different amorphization processes on trehalose in presence of limonene, preliminary DSC and gas-chromatographic analyses have been carried out on simple samples made of  $\beta$ -crystalline trehalose (TRE-b) mixed with limonene at different concentration.



Thermal properties of the samples obtained by addition of 2.7 and 11% w/v are reported in **Fig. 3.2.1**. The thermograms of pure limonene have been recorded under several experimental conditions (e.g. scanning rate, open or sealed cell), showing a continuous exponential rising of evaporation with increasing temperature up to the consumption of the liquid phase in the temperature range of 30°C to 100°C and 80 to 170 °C in agreement with data reported by Hazra et al., 2002 and Stephenson and Malanowski, 1987.

Although the general shape resemble those recorded in Fig. 3.2.1., these curves cannot fit any of pure limonene, because the difference in the morphology of the samples and the likely steric hindrance of TRE towards the phase change of the aroma (pure liquid vs entrapped one). Therefore, by comparison of the thermograms of the pure  $\beta$ -crystalline trehalose, the simple addition and mix with limonene determined only the appearance of a main and broad endothermal peak starting from 80°C and partly overlapping that one of the melting of the TRE-  $\beta$  occurring at ca 215°C (Cesàro et al., 2008). Finally, the thermal trend observed in the TRE-  $\beta$ +LIMO mixed samples is associated to the evaporation process of the added limonene, a process that, however, that may thus depend on the physical distribution of the aroma. This aspect will deserve further attention.

The retention of limonene in the TRE-Limo mixed samples was determined after 24 h of contact time and according to the standard procedure, both the surface and the encapsulated/retained (corresponding to the aroma found in the trehalose dissolved in the solvent after washing the crystals to evaluate the aroma present onto the surface) Limo in the matrix was evaluated. Results of the GC analysis confirmed that almost all added limonene was completely present at the surface of the TRE crystals. In general over 98.5% of the added aroma was present in the first hexane extract of the sugar-aroma samples and removed from the solid, crystalline trehalose and < 0.2% was found in the washed crystals; the lacking amount of the added volatile (ca. 1.3 %) could also be lost during the handling of the sample due to the high volatility of (R)-Limo. In the anhydrous  $\beta$ -crystalline state (TRE- $\beta$ ) with a monoclinic (P2<sub>1</sub>) crystal structure trehalose all the hydroxyl groups of the sugar moiety are involved in the network of

hydrogen bonds (Jeffrey and Nanni, 1985). Moreover, both the ring-oxygen atoms are involved in the hydrogen bond while the glycoside oxygen atom is not.

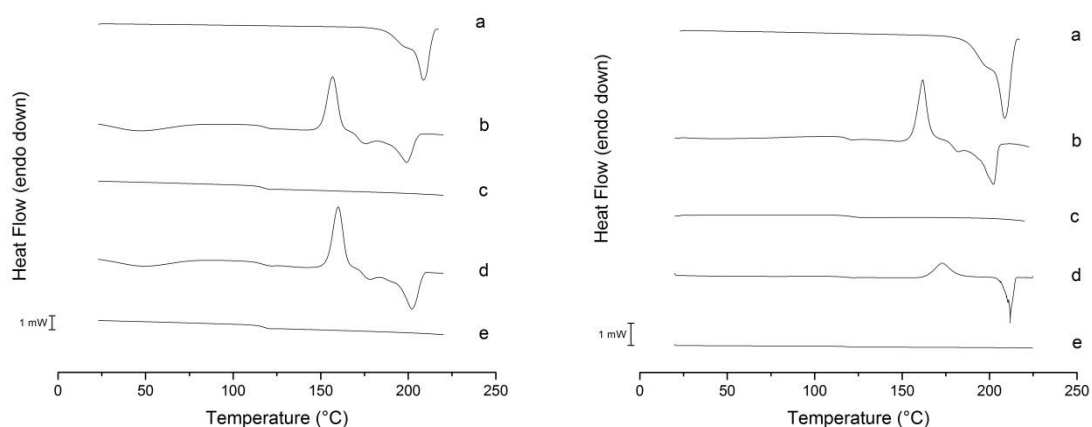
The highly structured crystalline matrix with no intermolecular spaces along with its relatively limited hydrophobicity could be implied in the lack of interaction with the limonene that, thus, could be easily removed upon contact with an apolar solvent.

### **3.2.2. Milling and comilling: preliminary investigations**

Amorphous matrices are generally obtained by the rapid undercooling of a melted crystal (Ediger et al., 2000). Among the alternative routes for solid state amorphization, is comprised milling that, when carried out at temperatures below the glass transition temperature ( $T_g$ ) of the corresponding liquid, has been shown to induce a non-thermal amorphization of a crystalline matrix (Willart et al., 2001).

A preliminary part of this study on milling of TRE-b was dedicated to evaluate different processing conditions on the amorphization of trehalose and to compare them when the same milling process was applied in presence of limonene (comilling).

**Figure 3.2.2** shows the DSC scan recorded upon heating in open pans of the anhydrous crystalline TRE-b before (initial) and after a milling treatment of 12 h. The effects of ball milling on the physical and functional properties of trehalose and in particular those related to the thermal behavior have been investigated and reported in the literature (Descamps et al. 2006; Willart et al., 2001).



**Figure 3.2.2.** Thermograms obtained upon heating at 5 K/min in open cells of milled TRE-b and comilled TRE-b\_(R)Limo for 12 h (A) and 15 h (B). a) crystalline TRE-b; b and c) initially anhydrous crystalline TRE-b after milling; d) and e) initially anhydrous crystalline TRE-b comilled with 2.7 % of (R)-Limonene b) and d) 1<sup>st</sup> scan; c) and e) 2<sup>nd</sup> scan (ball milling equipment: Pulverisette 7).

Upon milling, the thermogram of the TRE-b appears more complex than the initial crystalline states with a series of peaks that are relevant to characterize the physical properties of the processed sugar. In particular the following features can be observed:

- Presence of a broad endothermic peak in the temperature range between 30 and 100°C that can be clearly attributed to the water release (Willart et al., 2001);
- A small endotherm at 120°C that mimics the glass transition signature of that of the undercooled trehalose. It must be attributed to the glass transition of the amorphous trehalose obtained by milling of the anhydrous, crystalline TRE-β form.
- An exothermal peak from 150 °C and 190 °C that corresponds to the cold crystallization towards the thermodynamically stable anhydrous crystalline TRE-b. The recrystallization is a clear indirect feature of the amorphysation that has been induced by the mechanical stresses during milling of the crystalline TRE-b.
- A broad endothermic peak located between 200 and 220 associated to melting of the crystalline TRE-b. The enthalpy ( $\Delta H$  91.2 J·g<sup>-1</sup>) is higher than that of the cold crystallization and this indicates that under this process condition a

remaining crystalline fraction in the m-TRE is still present and thus, melts along with that formed during the induced cold crystallization (Willart et al 2001). The enthalpies of melting is found however lower than the enthalpy of melting of the initial, non-milled TRE ( $\Delta H = 154 \text{ J}\cdot\text{g}^{-1}$ ) and this indicates that in the samples analyzed a fraction of the saccharide has not crystallized upon heating.

The behavior observed by using commercial anhydrous TRE-b is in agreement with that reported in Willart et al (2001) on crystalline TRE-b of different manufacturing process and milled for 20 and 100 h. No further investigations have been carried out to evaluate the presence and quantify the entity of the crystalline fraction in the milled samples (eg. powder X-ray diffraction) as well as in those that will be obtained by comilling of the TRE-b in presence of limonene.

To obtain an index of the amorphous fraction on the milled (and comilled) samples, the ratio  $\Delta C_{pS}/\Delta C_{pTa}$  was determined where  $\Delta C_{pS}$  corresponds to the  $\Delta C_{pS}$  at the glass transition of the m-TRE (or co-mTRE) sample and  $\Delta C_{pTa}$  that of the  $\Delta C_{pS}$  at the glass transition of the fully amorphous TRE ( $0.65 \text{ J K}^{-1} \text{ g}^{-1}$ ). For the m-TRE samples reported in Figure 3.2.2 a value of ca. 0.98 was obtained by confirming the amorphization effect induced by the process.

In the same **Figure 3.2.2.** the thermograms of the comilled TRE\_(R)-Limo (co-m TRE\_(R)-Limo) obtained by an initial mix of TRE-b and 2.7 % of aroma processed for the same time is reported. A similar trend could be observed with small changes in the initial temperature of cold-crystallization and in the entity of the amorphous trehalose. In particular the presence of limonene delays the start of the cold crystallization but enhances it in terms of enthalpy. A significantly higher melting enthalpy was moreover observed. These results can be due to the effect of the limonene that differently entrapped/encapsulated in the amorphous matrix during milling could thus affect the mobility of the system in its rubbery state and the rate of state transitions of the mixed co-milled TRE-Limo system.

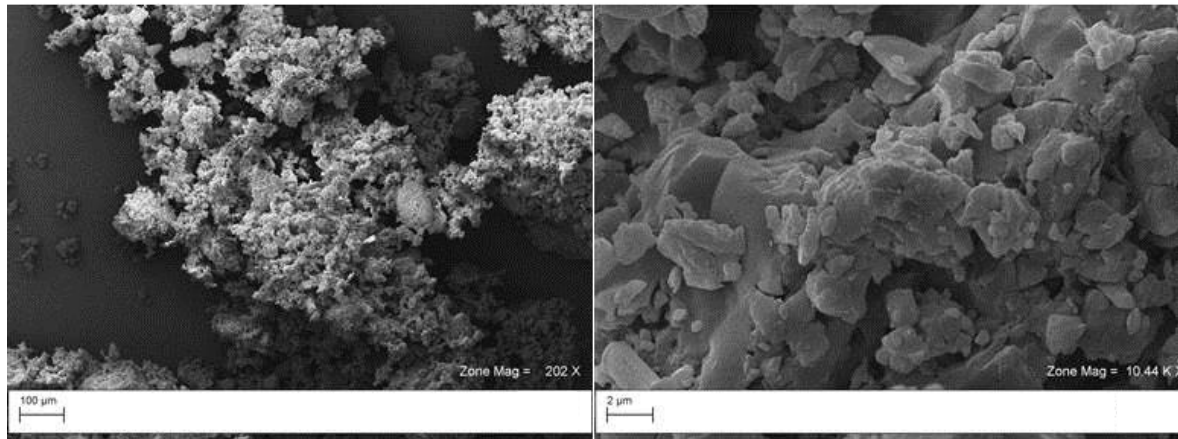
No main endothermal peaks corresponding to the aroma evaporation could be evidenced as, on the contrary, seen in the TRE-b mixed with (R)-Limo at the same concentration (Figure 3.2.1.). In the 12 h co-m TRE\_(R)limo samples, the aroma

content determined just after process was equal to 58 % in respect to the quantity added in the initial mix while onto the surface a limited amount (< 0.3 %) was found. This suggests that the limonene during the milling process becomes encapsulated or entrapped in the amorphous matrix of the trehalose and/or adsorbed in the porous matrix and/or dissolved and solubilized in the sugar matrix as well as in the residual even if limited water of the system.

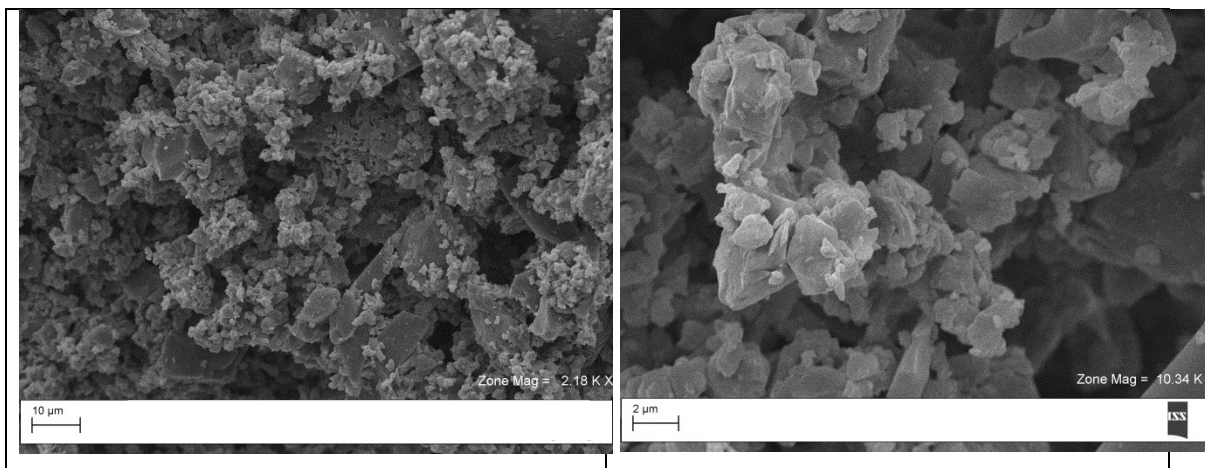
Sample	Glass transition		1st peak ( <i>exotherm, crystallization</i> )			2nd peak ( <i>endotherm, melting</i> )		
	Tg <sub>ons</sub> (°C)	Tg <sub>mid</sub> (°C)	Tc <sub>ons</sub> (°C)	Tc <sub>peak</sub> (°C)	ΔHc (J g <sup>-1</sup> )	Tm <sub>ons</sub> (°C)	Tm <sub>peak</sub> (°C)	ΔHm (J g <sup>-1</sup> )
TRE-b	-	-	-	-	-	202,6	207,8	154.2
m-TRE	114.3±0.2	119.7±0.2	150.6±1.0	156.8 ±0.8	74.3 ±1.0	175.9±0.8	199.1±0.3	91.2±1.7
co-m TRE- (R)Limo (2.7%)	110.8±0.3	119.2 ±1.0	156.2±1.2	163.2 ±1.1	93.7 ±1.5	203.7±1.1	208.5±1.0	115.6±0.6

Tg<sub>ons</sub>: Tg at onset point; Tg<sub>mid</sub>: Tg at mid ΔCp; Tc<sub>ons</sub>: initial temperature of re-crystallisation; Tc<sub>peak</sub>: max temperature of the crystallization peak; ΔHc: enthalpy of crystallization; Tm<sub>ons</sub>: initial temperature of melting; Tm<sub>peak</sub>: max temperature of the melting peak; ΔH<sub>m</sub>: enthalpy of melting

**Table 3.2.1.** DSC data of crystalline TRE-b, milled TRE-b and co-milled TRE-(R)-Limo processed for 12 h.



**Figure 3.2.3.:** Scanning Electron Microscopy (SEM) images of milled TRE (12 h) at different magnifications



**Figure 3.2.4.** Scanning Electron Microscopy (SEM) images of comilled TRE\_(R)-Limo (12 h) at different magnifications

In **Figure 3.2.3.** and **Figure 3.2.4.** the microstructure of the milled TRE and comilled TRE-(R)Limo samples undergone for the same processing time (12 h) is shown. A fine powder-like microstructure made of granules of micron- and submicron size and characterized by an irregular shape has been observed. Granules present a dense and compact structure that is associated to their glassy state. When TRE is comilled in presence of limonene, a similar structure is observed even if the presence of holes of submicron size can be also observed (**Figure 3.2.5**). While several studies have been carried out and results reported in the literature about the effects of milling and comilling on microstructural properties of metals and ceramics, no data have been reported so far on small saccharides and, in particular, on trehalose and this result highlights the importance of this evaluation.

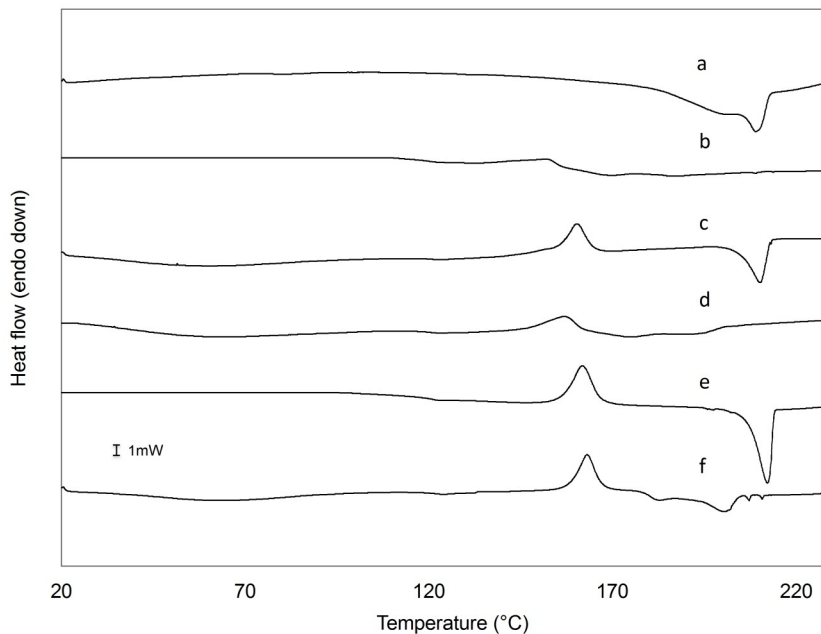
To our knowledge no previous studies of comilling of two molecular materials of different physical state (one solid, the other liquid) have been previously carried out under the conditions applied in this study (ball-milling, T below T<sub>g</sub> of the solid, dispersant material). The results of this preliminary study highlight effects on the thermal behavior and the microstructural features of the milled and comilled TRE due to the presence of limonene. This arises the need of further and deeper investigations to provide a deeper insight on the phenomena occurring during milling of TRE in

presence of a cosolute in liquid state like the limonene by taking into account in particular the following factors:

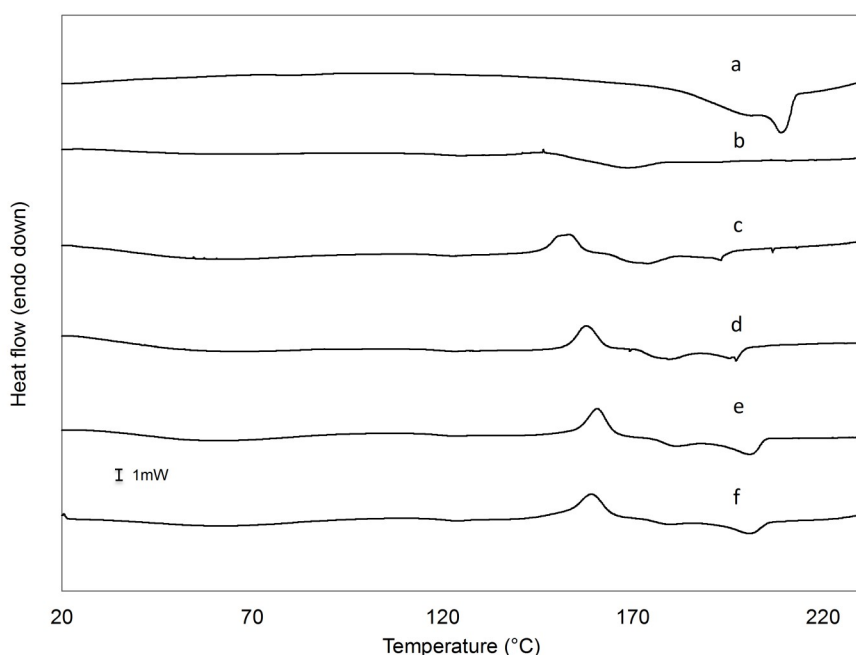
- Time of milling/comilling
- Concentration of limonene during comilling
- Type of saccharide

Each of these factors has been object of investigation, presented and discussed in the following sections of this study.

### 3.2.4 Effect of processing time of milling/comilling



**Figure 3.2.5.** Thermogramms (heating rate =  $5 \text{ K min}^{-1}$ ) of initially anhydrous TRE-  $\beta$  as a function of milling time. a) anhydrous TRE-  $\beta$ ; b) milled for 4 h; c) milled for 6 h; d) milled for 9 h; e) milled for 12 h; f) milled for 16 h (ball milling equipment: Pulverisette 6)



**Figure 3.2.6.** Thermograms (heating rate =  $5 \text{ K min}^{-1}$ ) of initially anhydrous TRE- $\beta$  in presence of (R)-Limonene (2.7 %w/w) as a function of comilling time. a) a) anhydrous TRE- $\beta$  mixed with 2.7% limonene (unprocessed sample); b) comilled for 4 h; c) comilled for 6 h; d) comilled for 9 h; e) comilled for 12 h; f) comilled for 16 h (ball milling equipment: Pulverisette 6).

The effect of milling time on anhydrous crystal TRE- $\beta$  has been investigated alone or in presence of a constant concentration of (R)-Limo (2.7 % w/w) (comilling).

In **Figure 3.2.5**. DSC scans obtained by heating (at 5 K/min) of anhydrous TRE-b subjected to different milling time from 2 up to 16 and in **Figure 3.2.6**. the scans obtained by comilling of initially anhydrous TRE-b in presence of 2.7% of (R)-Limo, are shown. The thermogram of the crystalline TRE-b is reported for comparison purposes in Figure 3.2.5.

The effect of the time was investigated by subjecting both the TRE-b and the initial mix of TRE-b + (R)-Limo up to 16 h that, based on the literature data (Willart et al., 2001) and the preliminary investigations was evaluated as sufficient to achieve an almost complete amorphization of the initial TRE-b.

The analysis of the thermograms obtained on samples at different process time and in presence/absence of the aroma compound highlights a progressive change of the physical properties of TRE at increasing milling time as well as the effect of limonene in the comilled samples by progressively and significantly affecting the TRE and TRE-Limo



systems thermal behavior. In **figure 3.2.7**, the change of the initial ( $a, T_{c/ons}$ ) and peak temperature ( $b, T_{c/peak}$ ) of the crystallization peak and the corresponding enthalpy ( $c, \Delta H_c$ ) as well as of the enthalpy of the melting peak ( $d, \Delta H_m$ ) are shown as a function of the milling/comilling time.

Overall, the results highlight the following:

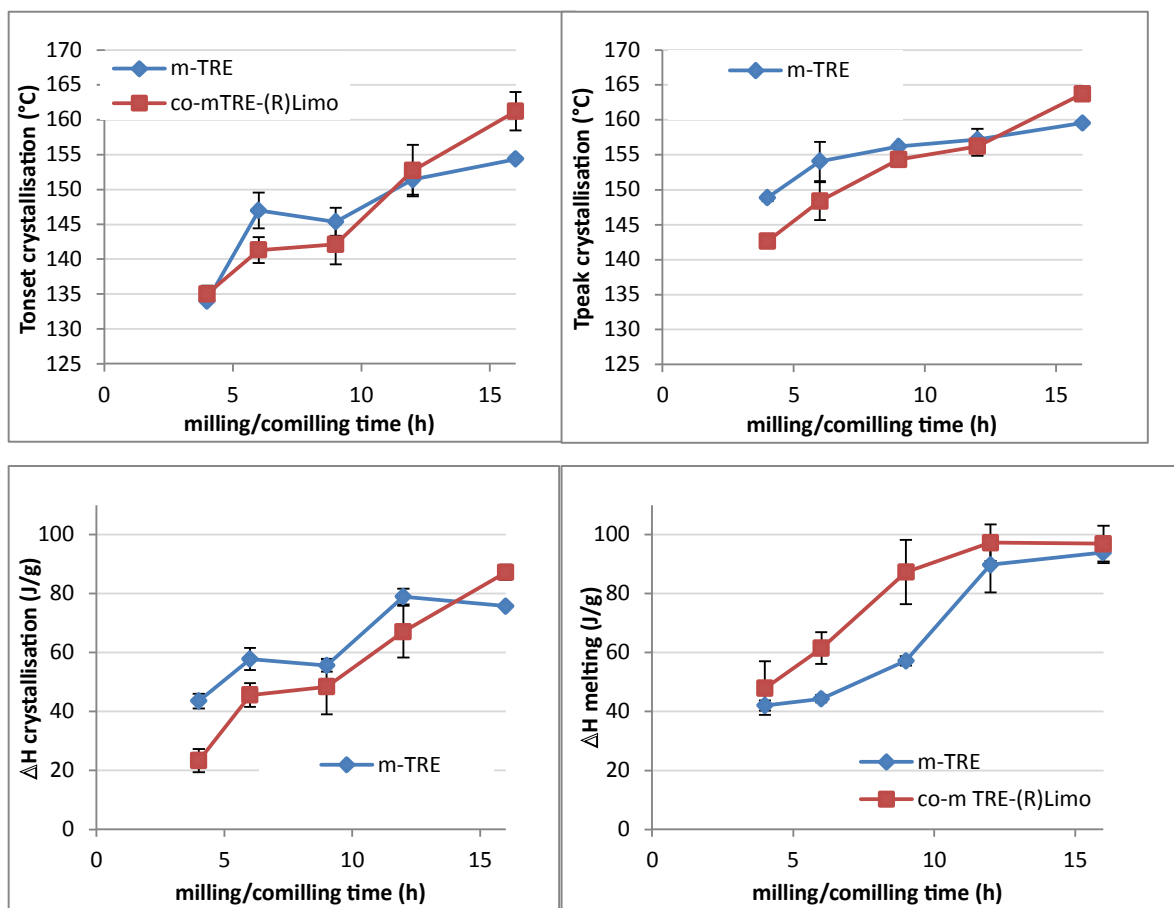
- In both the milled and comilled samples, a glass transition is evident at a temperature of ca. 120 °C also in the samples undergone to a short milling time (2-4 h). The temperature at which the glass-to-rubbery transition occurs ( $T_g$ ) results in all cases in the range 119 and 120°C and it is not significantly affected by both milling time and presence of limonene in the co-m TRE\_(R)-Limo samples. However, the presence of limonene affects the  $T_g$  onset of the comilled samples independently on the processing time. A decrease of the  $T_g$  onset from  $114 \pm 0,5$  °C (m-TRE) to  $110 \pm 0,9$  °C (co-m TRE) has been observed. This is related to the presence of limonene that, dispersed in the matrix, could affect the mobility of TRE while not modifying the overall temperature of the glass-to-rubbery state of the system.
- In all processed samples, a broad exothermal peak corresponding to cold-crystallization has been evidenced. The initial temperature ( $T_{c_{ons}}$ ) and the peak temperature of the crystallization ( $T_{c_{peak}}$ ) resulted differently affected by the milling/comilling time along with the  $\Delta H$  ( $\Delta H_c$ ) associated to this event. In particular at low milling time and up to 9-10 h of milling the presence of limonene delays the transition of the amorphized TRE ( $T_{c_{ons}} \text{ m-TRE} > T_{c_{ons}} \text{ co-m TRE}_{(R)}\text{Limo}$ ) and its entity but above 12 h the opposite behavior has been observed.
- A series of endothermic peaks have been evidenced in both milled and comilled samples at temperatures above 160°C. For samples milled at shorter processing time the peaks superimpose onto the right wing of the broad crystallization peak. Two main endothermic events are in general shown, one with a maximum at ca. 175-180°C and a second that progressively moves from

190 to 200-210°C. At increasing time, the entity of the latter increases while the opposite occurs for the former one. This thermal behavior may correspond to the simultaneous presence in the sample of TRE in different states and crystal polymorphs partly of the initial TRE-b, partly formed during the cold crystallization induced upon heating of the amorphized TRE during milling in presence/absence of (R)-Limo.

- The enthalpy of melting of the crystalline fraction in the comilled samples appears significantly higher than that observed in the milled-TRE samples at all processing time. This could correspond to a higher presence of crystalline TRE not amorphized during milling due to the presence of limonene.

The role of limonene in affecting the thermal behavior and physical state of comilled TRE has been further confirmed in following experiments to investigate a wider range of milling/comilling time whose results are summarized in **Table 3.2.2**. By taking as hypothesis that samples milled/comilled for 12 h have reached an almost complete amorphous state of TRE and that lower processing time determines a lower crystalline-to-glass transition (Lefort et al., 2004), these results suggest that limonene may play a different role in the milled/comilled TRE under the solid state amorphization induced by milling depending on the ratio of the amorphous to crystalline fractions.

When a the crystalline state is prevalent (low milling times) it hinders the cold crystallization, while when TRE is completely amorphized (milling time > 12 h) , the presence of limonene makes it easy and favors its evolution.



**Figure 3.2.7:** Change of  $T_{onset}$  and  $T_{peak}$  crystallization,  $\Delta H$  crystallization and  $\Delta H$  melting of the crystalline fraction of milled TRE (m-TRE) and comilled TRE-(R)Limo as a function of milling time (Pulverisette 6).

Sample (milling/comilling time)	1 <sup>st</sup> peak (exotherm, crystallization)			2 <sup>nd</sup> peak (endotherm, melting)					
	$T_{C_{ons}}$ (°C)	$T_{C_{peak}}$ (°C)	$\Delta H_c$ (J g <sup>-1</sup> )	Peak 1			Peak 2		
				$T_{m_{ons}}$ (°C)	$T_{m_{peak}}$ (°C)	$\Delta H_m$ (J g <sup>-1</sup> )	$T_{C_{ons}}$ (°C)	$T_{C_{peak}}$ (°C)	$\Delta H_c$ (J g <sup>-1</sup> )
m-TRE (2h)	137.2 ±1.5	147.2 ±1.3	30.8 ±2.0	157.3 ±1.0	166.1 ±0.5	13.0 ±1.6	177.4 ±0.9	190.2 ±1.2	14.9 ±1.9
co-m TRE-(R)Limo (2.7%) (2 h)	133.7 ±0.7	143.7 ±1.0	22.1 ±1.8	155.2 ±0.7	165.2 ±0.8	19.9 ±1.9	175.7 ±1.3	184.2 ±1.1	6.84 ±2.1
co-m TRE-(R)Limo (2.7%) (18 h)	160.9 ±0.5	170 ±1.0	83.1 ±0.7	-	-	-	205.1 ±0.7	211.2 ±0.5	86.0 ±1.5
co-m TRE-(R)Limo (11.0 %) (2h)	152.6 ±0.9	159.8 ±0.6	84.7 ±1.0	-	-	-	203.0 ±1.0	210.0 ±0.8	106.8 ±0.9

$T_{g_{ons}}$ :  $T_g$  at onset point;  $T_{g_{mid}}$   $T_g$  at mid  $\Delta C_p$ ;  $T_{c_{ons}}$ : initial temperature of re-crystallisation;  $T_{c_{peak}}$ : max temperature of the crystallization peak;  $\Delta H_c$ : enthalpy of crystallization;  $T_{m_{ons}}$ : initial temperature of melting;  $T_{m_{peak}}$ : max temperature of the melting peak;  $\Delta H_m$ : enthalpy of melting. Mean  $\pm$  standard deviation. <sup>a,f</sup>: different letters means significant difference ( $p < 0.05$ ) of the means in the same column

**Table 3.2.2.** DSC data of crystalline TRE-b, milled TRE-b and co-milled TRE-(R)-Limo processed for 12 h.

Limonene, being liquid at the milling temperature, is expected to act as plasticizer and thus, to modify the  $T_g$  of the amorphized system according to the Gordon-Taylor equation (Roos, 1995). The no variation of the  $T_g$  of the TRE\_(R)Limo in respect to the pure amorphous TRE obtained by quenching seems suppose that under our process and formulation conditions a “liquid-solid dispersion” has been obtained where TRE acts as main dispersant of nano-droplets of limonene with a phase separation difficult to detect via calorimetric analysis. When only one  $T_g$  is detected the solid dispersion is usually assumed to be a homogeneous solution that may contain segregated amorphous or liquid domains that are too small to be detected and to affect the main thermal properties of the continuous phase (Huang and Dai, 2014). Separated domains of approximately 100  $\mu\text{m}$  in size has been evaluated by confocal Raman microscopy as limit for a solid dispersions (Qian et al., 2010) while other studies refer to a lower size (30 nm) (Newman et al., 2008). Some authors on studies carried out on pharmaceutical matrices explained this effect as due to a no change of the overall “free volume” of the amorphous system (Crowley et al., 2002).

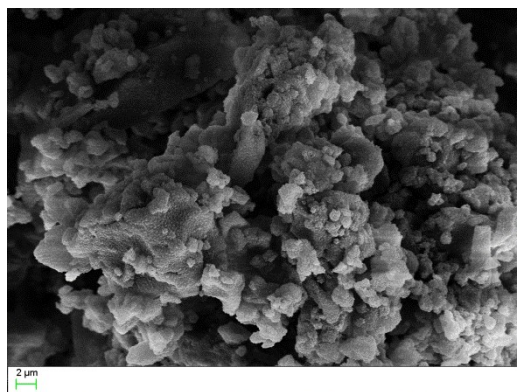
The evolution of the microstructural properties of the comilled TRE-(R)Limo (2.7%) samples at increasing milling time is reported in **Figure 3.2.8**. The sequence of the images highlights the progressive change of the physical properties of the trehalose-limonene mixed system upon the high-energy stress applied during co-milling.

The 2 h-processed sample shows attrition of the larger particles as observed also by the appearance of small particles on the surface of the larger one. The 18 h-comilled sample presents, on the contrary, a large number of fine particles and granules resulting from the process-induced disorder of the milling (York, 1983). The particle surface evidences also the presence of shear-like bands on the particle surface that is consistent with the way glasses tend to crack upon deformation induced by the high energy applied during the process.

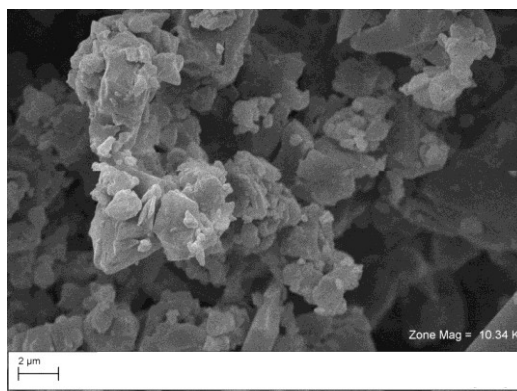
The micrographs of the 2, 12 and 18 h of comilling are characterized also by a different brightness that could be due to the progressive change of the trehalose from the crystalline-like state to the amorphous one. The presence of small, submicron holes is

evidenced in the samples processed for times above 12 h where the aroma could be finely dispersed and thus, entrapped.

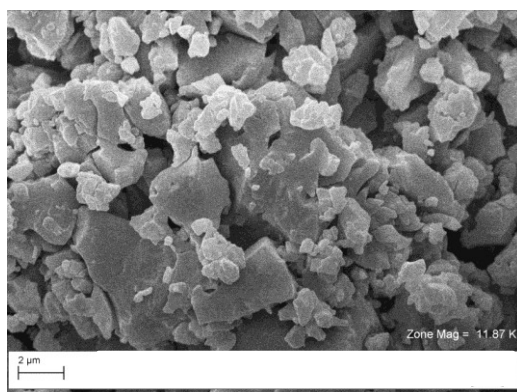
These images seem thus to confirm the hypothesis that in our conditions a liquid-solid dispersion of limonene in amorphous TRE has been produced.



**a) com-TRE\_(R)-Limo (2.7 %, 2 h)**

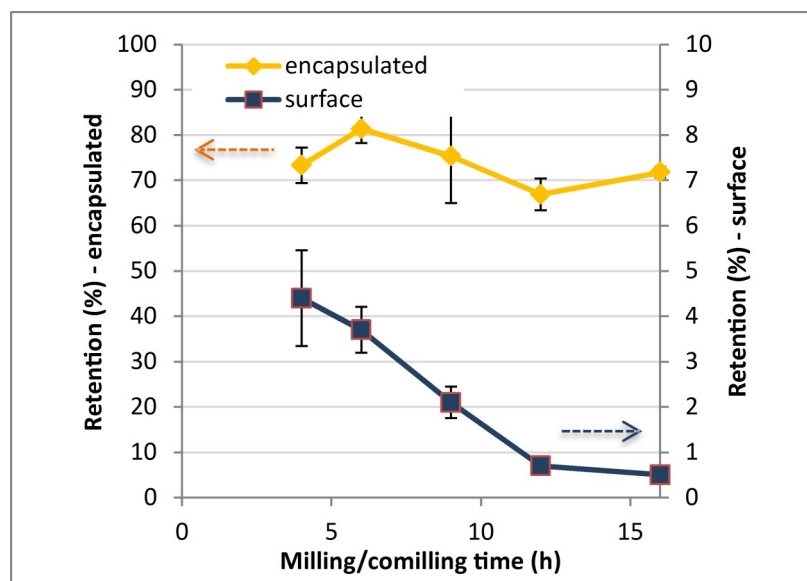


**b) com-TRE\_(R)-Limo (2.7 %, 12 h)**



**c) com-TRE\_(R)-Limo (2.7 %, 18 h)**

**Figure 3.2.8.** Morphology of comilled TRE-(R)Limo (2.7%) samples obtained after different processing time (2, 12 and 18 h) as observed by SEM. Magnification for each image is given on each SEM image. a): 2 h; b): 12 h); c) 18 h.



**Figure 3.2.9.** Retention of limonene in comilled TRE-(R)-Limo (2.7%) as a function of processing time (% on the aroma quantity added in the initial sugar-aroma mixture). Data are reported distinctly for the aroma encapsulated in the matrix and that determined onto the surface (milling equipment: Pulverisette 6).

In **Figure 3.2.9** the effect of comilling time on the ability of the developed matrix to retain limonene added in the initial mix at a concentration of 2.7%. Analyses have been carried out by a procedure aimed to evaluate both the aroma fraction retained in the progressively developing amorphous matrix and defined as “encapsulated” and that still present in the sample but present onto the surface of the milled powder.

Data reported evidence that the comilled TRE-(R) Limo systems present a rather high ability to retain the aroma within the progressively forming amorphous matrix with percentages higher than 55% for all the comilled samples independently on the processing time. These values are significantly higher than those observed in the spray-dried samples obtained starting from an aqueous system containing similar aroma concentration (2.5%). Comilling time, despite the lack of statistical significance due to a large variability of the data of the 9h-comilled samples, seems to have some effect on the retention by increasing the retention at lower milling time (i.e. matrices only partly amorphous) up to 6 h and then causing a decrease until an equilibrium value of limonene retention is obtained after 12 h. Average data of limonene retention obtained by several independent experiments carried out by the two different ball

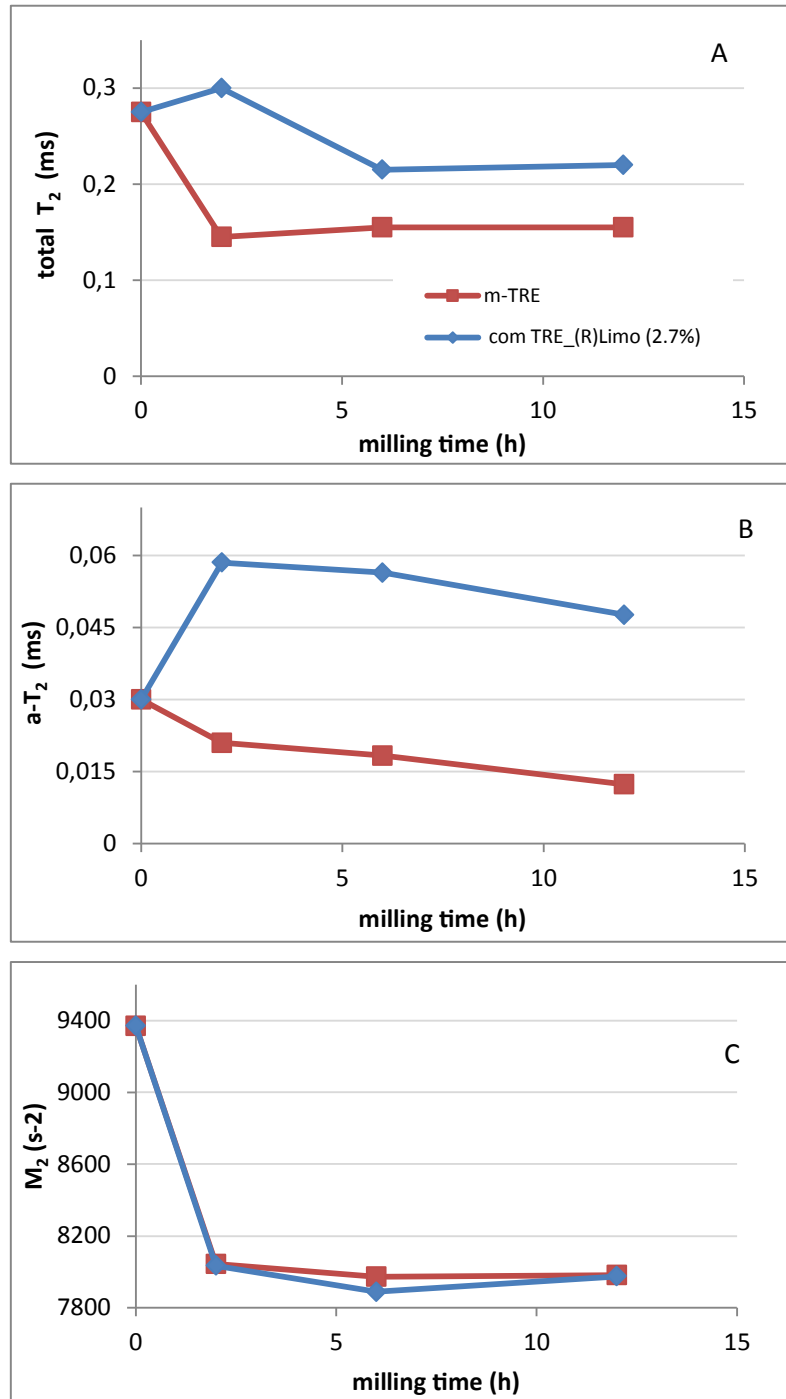
milling systems (high energy and conventional) of comilled TRE-(R) Limo samples that based processed for 12 h are in the range of 58 % and 68%.

Moreover, the percentage of limonene present onto the surface of the comilled TRE powders showed a progressive decrease at increasing processing time, corresponding to a higher retention in the amorphous matrix.

In all cases, however, the sum of the limonene encapsulated and present in the surface does not reach the initial added aroma in the mix and this has to be related to losses occurring during the milling process as well due to some aroma adhering onto the miller chamber. At the end of the process part of the comilled saccharide results tightly attached onto the milling chamber wall and bottom and likely this could be caused by liquid limonene that determines the formation of a viscous layer where part of the sugar sticks and that will be no more removed even after long processing time.

NMR analysis has been carried out on the milled and comilled TRE samples and average and total relaxation time ( $\rho\text{-}T_2^*$  and  $t\text{-}T_2^*$ ) and  $M_2$  as a function of processing time were determined. All the mobility parameters resulted to vary upon milling/comilling time and significantly affected at all processing time by the presence of the aroma in the system (**Figure 3.2.10**).

$M_2$  is significantly affected by the milling time as a consequence of the crystalline-to-amorphous solid state transition induced by the process and this causes change in the molecular interactions of the molecules. The presence of limonene at low milling time seems to initially differently contribute to the keep a higher strength in the interactions among the TRE matrix molecular of the matrix but when the system is completely amorphized, no differences could be significantly observed.



**Figure 3.2.10:** Average (A) and total (B) relaxation time ( $a-T_2^*$  and  $t-T_2^*$ ) and second moment values ( $M_2$ ) (C) of milled TRE and comilled TRE\_(R)Limo (2.7%) as a function of the processing time.

As regards the relaxation time, both  $a-T_2^*$  and  $t-T_2^*$  a significant change occurred in respect to the initial crystalline TRE-b. Both the total and the average relaxation time of the milled TRE resulted lower than that of the TRE-b reflecting the physical changes occurred during the amorphization process. While the  $t-T_2$  index does not change upon



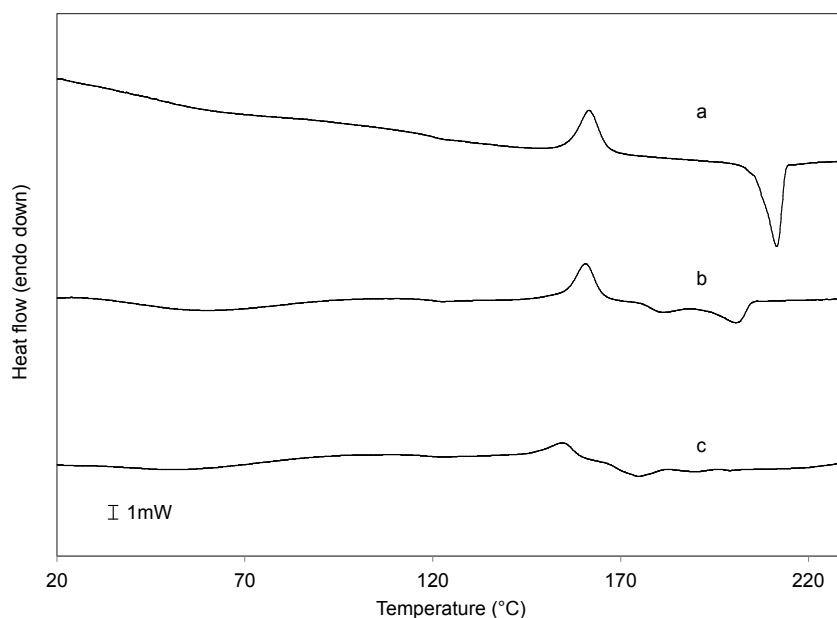
milling time the  $a-T_2^*$  tends to progressively decrease and this likely reflect the corresponding changes in the physical properties of the milled samples and the progressive amorphization.

As regards the comilled TRE in presence of limonene, higher  $a-T_2^*$  values were find in respect to the initial crystalline TRE-b and the milled TRE at all processing time with a decreasing trend within the 12 h of processing applied to the system similar to that found in the milled TRE. These results could be associated to both the presence of limonene that increases the mobility of the system and the progressive amorphization of the matrix.

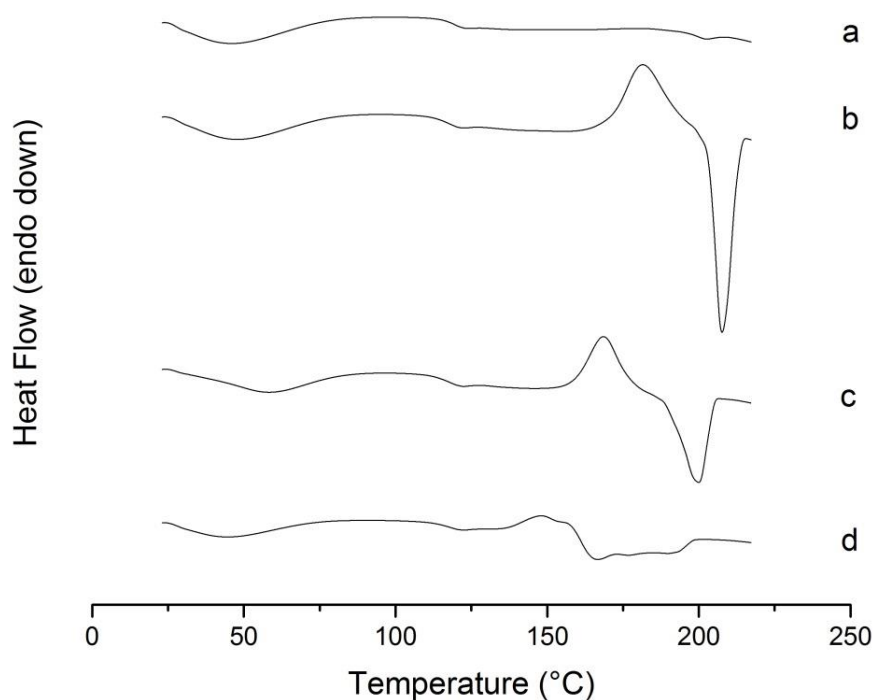
### 3.2.4. Effect of the limonene concentration

The initial quantity of aroma present in the TRE-Limonene mix before comilling affected significantly the crystalline-to-glassy state evolution of the TRE upon milling.

In **Figure 3.2.11** the DSC scans of milled trehalose and comilled TRE\_(R)Limo at increasing concentration up to 11 % w/w of initial aroma in the system, all processed for 12 h are shown.



**Figure 3.2.11.** Thermograms (heating rate:  $T = 5$  K) of comilled TRE\_(R)Limo samples made of initially anhydrous TRE-  $\beta$  and different (R)-Limo for 6/12 h processing time. a) milled TRE, 0% (R)Limo; b) co-m TRE\_(R)Limo 2.7%w/w; c) co-m TRE\_(R)Limo 11.0 %w/w. (ball milling equipment: Pulverisette 6).



**Figure 3.2.12.** Thermograms (heating rate:  $T = 5 \text{ K}$ ) of comilled TRE\_(R)Limo samples made of initially anhydrous TRE-  $\beta$  and different (R)-Limo of for 12 h processing time. a) milled TRE, 0% (R)- Limo; b) co-m TRE\_(R)Limo (2.7%w/w); c) co-m TRE\_(R)Limo (5.4 %w/w); d) co-m TRE\_(R)-Limo (11.0 %w/w). (ball milling equipment: Pulverisette 7).

The increase of the aroma concentration determined a progressive and significant decrease of both the temperature at which cold crystallization ( $T_c$ ) and melting ( $T_m$ ) occurs and of the entity of the same thermal phenomena in respect to those evidenced in the comilled systems at the 2.7 % initial concentration of limonene. This has been evidenced at both 6, 12 and 18 h of milling time and concentrations up to 11 % of initial added limonene (**Table 3.2.3.**).

At the same comilling time, at increasing aroma added in the initial mix a decrease of the  $\Delta C_p$  is also observed, index of the lower amorphization of TRE upon milling. The increase of the liquid phase (i.e limonene) in the system during ball milling could limit the effect of the high energy stresses induced during the milling and reduce the corresponding effect on the amorphization effect.

Sample			Glass transition			1 <sup>st</sup> peak (exotherm, crystallization)			2 <sup>nd</sup> peak (endotherm, melting)		
	Time * (h)	(R)-Limo (%)	T <sub>g_ons</sub> (°C)	T <sub>g_mid</sub> (°C)	ΔCp (J g <sup>-1</sup> °C <sup>-1</sup> *)	T <sub>c_ons</sub> (°C)	T <sub>c_peak</sub> (°C)	ΔH <sub>c</sub> (J g <sup>-1</sup> )	T <sub>m_ons</sub> (°C)	T <sub>m_peak</sub> (°C)	ΔH <sub>m</sub> (J g <sup>-1</sup> )
m-TRE	6	0	113.5	120.1 <sup>a</sup>	0.37 <sup>e</sup>	137.1 <sup>d</sup>	148.5 <sup>b</sup>	38.5 <sup>d</sup>	159.1 <sup>c</sup>	166.4 <sup>c</sup>	34.3 <sup>f</sup>
Co-m	6	2.7	110.7	118.8 <sup>b</sup>	0.54 <sup>c</sup>	138.6 <sup>d</sup>	144.3 <sup>d</sup>	58.0 <sup>c</sup>	153.9 <sup>d</sup>	165.4 <sup>c</sup>	75.1 <sup>d</sup>
TRE_(R)Limo	6	11.0	110.4	117.6 <sup>b</sup>	0.49 <sup>d</sup>	130.7 <sup>e</sup>	141.7 <sup>d</sup>	28.8 <sup>d</sup>	156.5 <sup>c</sup>	164.1 <sup>c</sup>	28.4
m-Treha	12	0	110.9	117.9 <sup>b</sup>	0.65 <sup>b</sup>	148.7 <sup>c</sup>	155.3 <sup>c</sup>	68.9 <sup>b</sup>	175.7 <sup>b</sup>	199.4 <sup>b</sup>	72.9 <sup>d</sup>
Co-m	12	2.7	111.7	118.9	0.63 <sup>b</sup>	149.4 <sup>bc</sup>	154.2 <sup>c</sup>	71.3 <sup>b</sup>	199.2 <sup>a</sup>	206.9 <sup>a</sup>	98.3 <sup>b</sup>
TRE_(R)Limo	12	11.0	111.0	117.9 <sup>b</sup>	0.59 <sup>a</sup>	143.1 <sup>b</sup>	151.1 <sup>b</sup>	28.3 <sup>c</sup>	166.5 <sup>a</sup>	170.7 <sup>c</sup>	58.7 <sup>e</sup>
Co-m	18	2.7	111.5	116.7 <sup>c</sup>	0.72 <sup>a</sup>	160.9 <sup>a</sup>	170.1 <sup>a</sup>	82.1 <sup>a</sup>	205.1 <sup>a</sup>	211.2 <sup>a</sup>	85.9 <sup>c</sup>
TRE_(R)Limo	18	11	110.6	116.6 <sup>c</sup>	0.64 <sup>b</sup>	152.6 <sup>b</sup>	159.8 <sup>b</sup>	84.7 <sup>a</sup>	203.2 <sup>a</sup>	210.0 <sup>a</sup>	106.8 <sup>a</sup>

T<sub>g\_ons</sub>: T<sub>g</sub> at onset point; T<sub>g\_mid</sub>: T<sub>g</sub> at mid ΔCp; T<sub>c\_ons</sub>: initial temperature of re-crystallization; T<sub>c\_peak</sub>: max temperature of the crystallization peak; ΔH<sub>c</sub>: enthalpy of crystallization; T<sub>m\_ons</sub>: initial temperature of melting; T<sub>m\_peak</sub>: max temperature of the melting peak; ΔH<sub>m</sub>: enthalpy of melting. <sup>a-f</sup>: different letters means significant difference (p<0.05) of the means in the same column

**Table 3.2.3.** Thermal data obtained from DSC scans of milled TRE and comilled TRE\_(R)-Limo for different time (6, 12, 18 h) and prepared with increasing aroma content.

This effect has been also confirmed when comilling for 12 h at increasing initial aroma concentrations has been carried out by using homemade crystalline trehalose-β (Figure 3.2.12). This disaccharide, after 12-h milling process, showed, differently to those obtained in the already discussed experiments carried out for similar processing time, a calorimetric trend of an amorphous system unable to undergo crystallization in the temperature range between T<sub>g</sub> and T<sub>m</sub>, typical of a glassy matrix prepared by cooling abruptly from a melting status (Sussich & Cesàro, 2008). Moreover, in this case, when comilled with a low concentration of limonene, both cold crystallization and melting of the amorphous fraction was observed with no main effects on the T<sub>g</sub> of the comilled system. At increasing initial content of (R)-Limo in the initial system a decrease of the temperatures at which both cold crystallization and melting occurs. The increased kinetics of the transition phenomena (crystallization and melting) is generally due to the presence of a plasticizer that, however, is expected also to affect the T<sub>g</sub> by inducing its decrease and/or an increase of the ΔCp (Le Meste et al., 2002).

In these systems the increased limonene added in the initial TRE-aroma mix causes a decrease of the  $\Delta C_p$  at all the processing time studied.

	Glass transition			1 <sup>st</sup> peak (exotherm, crystallization)			2 <sup>nd</sup> peak (endotherm, melting)		
	Tg <sub>ons</sub> (°C)	Tg <sub>mid</sub> (°C)	$\Delta C_p$ (J g <sup>-1</sup> °C <sup>-1</sup> )	T <sub>ons</sub> (°C)	T <sub>peak</sub> (°C)	$\Delta H_c$ (J g <sup>-1</sup> )	T <sub>ons</sub> (°C)	T <sub>peak</sub> (°C)	$\Delta H_m$ (J g <sup>-1</sup> )
TRE- $\beta$	-	-	-	-	-	-	202.0 <sup>a</sup>	207.2 <sup>a</sup>	89.7 <sup>a</sup>
m-TRE	115.2 <sup>a</sup>	119.3	0.69 <sup>a</sup>	nd	nd	nd	196.3 <sup>b</sup>	202.2 <sup>b</sup>	1.9 <sup>e</sup>
com-TRE_(R)-Limo (2.7 %)	112.3 <sup>b</sup>	117.0	0.64 <sup>b</sup>	170.5 <sup>a</sup>	181.5 <sup>a</sup>	52.9 <sup>a</sup>	203.4 <sup>a</sup>	207.7 <sup>a</sup>	65.4 <sup>b</sup>
com-TRE_(R)-Limo (5.2 %)	112.7 <sup>b</sup>	118.8	0.55 <sup>c</sup>	158.4 <sup>b</sup>	168.6 <sup>b</sup>	38.1 <sup>b</sup>	191.0 <sup>c</sup>	199.9 <sup>b</sup>	41.5 <sup>d</sup>
com-TRE_(R)-Limo (11.0 %)	112.3 <sup>b</sup>	118.8	0.57 <sup>c</sup>	137.9 <sup>c</sup>	147.5 <sup>c</sup>	6.1 <sup>c</sup>	158.2 <sup>d</sup>	166.2 <sup>c</sup>	60.4 <sup>b</sup>

Tg<sub>ons</sub>: Tg at onset point; Tg<sub>mid</sub>: Tg at mid  $\Delta C_p$ ;  $\Delta C_p$  at glass transition; Tc<sub>ons</sub>: initial temperature of re-crystallisation; Tc<sub>peak</sub>: max temperature of the crystallization peak;  $\Delta H_c$ : enthalpy of crystallization; Tm<sub>ons</sub>: initial temperature of melting; Tm<sub>peak</sub>: max temperature of the melting peak;  $\Delta H_m$ : enthalpy of melting. <sup>a-f</sup>: different letters means significant difference (p<0.05) of the means in the same column

**Table 3.2.4.** Thermal data obtained from DSC scans of milled TRE and comilled TRE\_(R)-Limo for different time (6, 12, 18 h) from TRE-b prepared according to Sussich et al. (2002) at increasing aroma content.

The increased mobility of the amorphous matrices prepared in presence of higher limonene concentration has been demonstrated also by NMR analysis and the change of the average relaxation time ( $T_2$ ) (**Figure 3.2.13.**). For samples treated at the same comilling time (6 or 12 h) the co-presence of 11% of limonene led to a significant increase of the average relaxation time (a- $T_2$ ) also in respect to the comilled TRE-Limo samples prepared with lower aroma concentration. The a- $T_2$  is an index of molecular mobility and increases when proton mobility increases and the higher dispersed limonene in the amorphous matrix could explain this result.

On the contrary, the second moment ( $M_2$ ) resulted only partially affected by the higher presence of the aroma and only at the shorter comilling time being more significant the main crystal-to-glass transformation. This could be related to the micro- and nano-dispersion state of the limonene in glassy, powder-like matrix that do not seem to affect the molecular interactions in the progressively physically changing matrix and its

rigidity. This could confirm the hypothesis that limonene could become entrapped as micro- and nano-dispersed droplets in the amorphous matrix in the free volume of the amorphous TRE without affecting significantly the strength of the interactions between the amorphous TRE particles.

Milling/comilling time (hours)	Limonene % in the initial system	(R)-Limonene concentration in comilled TRE_(R)-Limo powders (ppm)		Limonene Retention (% in respect to initial)	
		Encapsulated	Surface	Encapsulated	Surface
12	2.7	$17.2 \cdot 10^3$ <sup>e</sup>	$0.142 \cdot 10^3$ <sup>f</sup>	65.2	5.2
	5.4	$28.8 \cdot 10^3$ <sup>c</sup>	$1.24 \cdot 10^3$ <sup>d</sup>	55.3	2.3
	11	$23.1 \cdot 10^3$ <sup>d</sup>	$0.253 \cdot 10^3$ <sup>e</sup>	20.9	1.7
	14	$18.0 \cdot 10^3$ <sup>e</sup>	$57.2 \cdot 10^3$ <sup>b</sup>	15.9	40.9
18	2.7	$21.9 \cdot 10^3$	$0.252 \cdot 10^3$	81.1	0.9
	11	$51.6 \cdot 10^3$ <sup>a</sup>	$2.93 \cdot 10^3$ <sup>c</sup>	43.0	4.2
	24	$35.9 \cdot 10^3$ <sup>b</sup>	$168 \cdot 10^3$ <sup>a</sup>	14.9	70.2

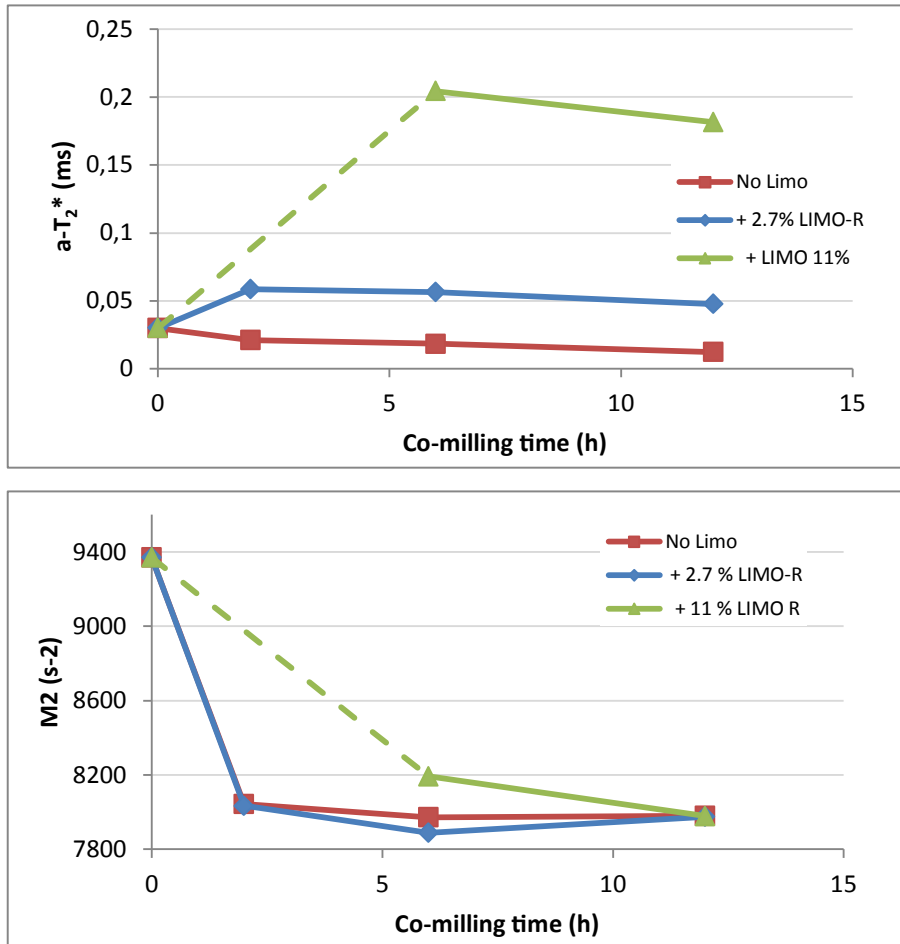
<sup>a-f</sup>: different letters means significant difference ( $p < 0.05$ ) of the means in the same column

**Table 3.2.5.:** Concentration of (R)-limo (ppm) and retention (R, % in respect to initial) in the comilled TRE-(R)Limo samples obtained by systems prepared with different initial aroma concentration and processed by ball milling for different time.

In general at higher initial quantity of limonene in the system and longer comilling time, an increase of the encapsulated aroma in the TRE amorphous matrix was observed (**Table 3.2.5**) even if, expressed on the initial concentration the retention as % to the initial resulted to decrease. However, when an excess of limonene was added (24%) the retained/encapsulated limonene in the 18 h co-m TRE was lower than that found in the sample prepared with lower concentration (11%) where on the contrary a large amount of “free” aroma, not encapsulated was found, suggesting a “cushion” effect of the limonene during processing limiting the encapsulating effect of the high energy milling process.

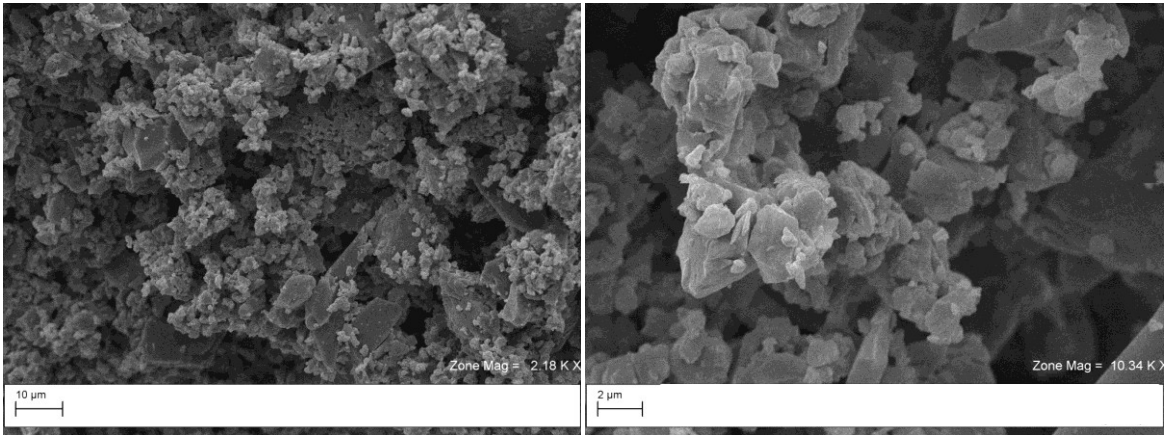
However, under the same conditions, an increased amount of limonene present in the amorphous matrix was observed and this evidences the main role of the milling/comilling processing time in affecting the encapsulation effect of a liquid cosolute in liquid state and its dispersion/dissolution.

To better investigate the potential of the amorphous comilled TRE matrix to encapsulate/retain higher amounts of limonene, longer comilling time need to be investigated and part of further investigations.

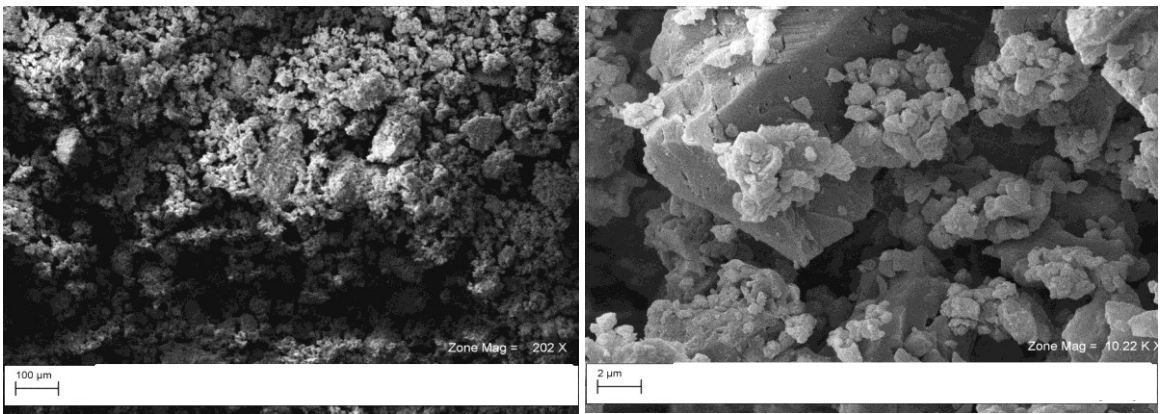


**Figure 3.2.13.** Variation of the average relaxation time ( $a_2T^*$ ) and second moment ( $M_2$ ) of milled TRE (no-limo) and comilled TRE at two different concentrations (2.7 and 11.0 %) of R-Limonene..

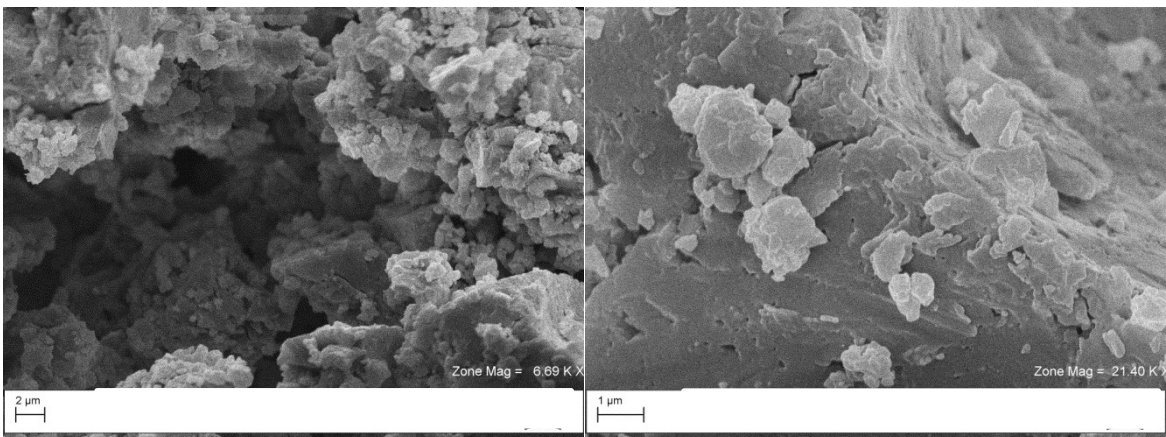
The increased added quantity of (R)-limonene in the initial trehalose-aroma mix affected also significantly the microstructural properties of the comilled systems (**Figure 3.2.14**). The comilled systems matrices are characterized by the significant presence of submicron and nano-holes finally dispersed in the granules of different size. Overall the higher quantity of limonene made the matrix more plastic and aggregate likely due to the high presence of free, not-encapsulated limonene and a lower number of submicron size granules are observed.



**Co-M TRE-(R)-Limo (2,7 %) (12 h)**



**Co-M TRE-(R)-Limo (11,0 %) (12h)**



**Co-M TRE\_(R)-Limo (11,0 %) (18h)**

**Figure 3.2.14:** Morphology of comilled TRE-(R)Limo (2.7% and 11.0%) samples obtained after different processing time (12 or 18 h) as observed by SEM. Magnification for each image is given on each SEM image.

### 3.2.5. Dissolution (mix) versus dispersion (comilling)

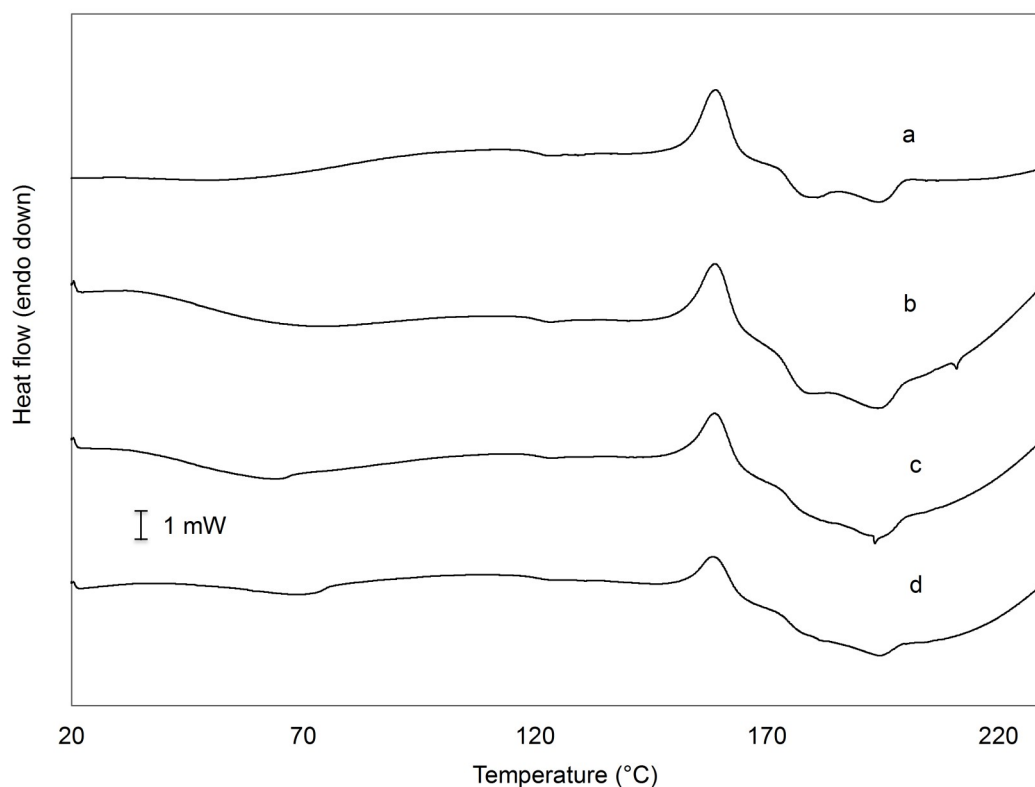
The application of comilling treatments on systems made of TRE and (R)-Limo is resulting to determine the formation of a liquid-in-solid dispersion system where limonene becomes entrapped in submicron and nano-size droplets. Additional processes implied in the retention in the amorphous TRE matrix could include also adsorption onto the powder particles and a solvation effect of the liquid aroma in the inter-particle space of the system.

On these premises it was our intention to evidence, if any, the effect of the presence of limonene acting as “solvent” on the thermal properties of milled, glassy TRE by the preparation of samples made of 12 h-milled TRE added, by mixing, with different concentrations of (R)-Limo. This could better allow to understand if and how comilling is affecting the dispersion state of the liquid (R)-Limonene in the matrix and the corresponding physical and thermal properties of glassy comilled TRE\_(R)Limo.

The TRE\_(R)-Limo mix at increasing aroma concentrations showed significantly different trends and thermal properties (**Figure 3.2.15**) than the systems made by comilling TRE and (R)-Limonene at the same aroma concentration. Above the glass transition and cold crystallization events, the thermogram becomes more complex: the melting of the crystalline fraction and corresponding peaks are partially superimposed to a main and wide endotherm that recovers its trend at temperatures higher than 220°C.

This behavior could be related to the simultaneous occurrence of endothermic thermal processes other than those referred to the melting of the crystalline trehalose like the limonene evaporation. This has been confirmed also by the significant difference in weight before and after thermal analysis of the samples added with limonene corresponding to a likely main loss of limonene.





**Figure 3.2.15.** Thermograms (heating rate:  $T = 5 \text{ K}$ ) of 12 h-milled TRE alone (a) and after mixing with increasing concentrations of (R)-Limo (b) + (R)Limo 2.7%w/w; c) + (R)Limo 5.2; d) + (R)Limo 11.0 %w/w (ball milling equipment: Pulverisette 6).

Miling/co -m time (h)	(R)- Limo (%)	Glass transition			1 <sup>st</sup> peak (exotherm, crystallization)			2 <sup>nd</sup> peak (endotherm, melting)		
		T <sub>g_ons</sub> (°C)	T <sub>g_mid</sub> (°C)	$\Delta C_p$ (J g <sup>-1</sup> °C <sup>-1</sup> )	T <sub>ons</sub> (°C)	T <sub>peak</sub> (°C)	$\Delta H_m$ (J g <sup>-1</sup> )	T <sub>ons</sub> (°C)	T <sub>peak</sub> (°C)	$\Delta H_m$ (J g <sup>-1</sup> )
12h	0	114.3 <sup>b</sup>	119.7	0.63 <sup>a</sup>	148.7	155.3	66.0 <sup>b</sup>	168.8	175.4 <sup>c</sup>	62.9 <sup>b</sup>
12h	2.7	116.7 <sup>a</sup>	119.3	0.50 <sup>c</sup>	149.5	154.3	71.3 <sup>a</sup>	168.2	196.9 <sup>a</sup>	86.3 <sup>a</sup>
12h	5.2	117.4 <sup>a</sup>	120.0	0.41 <sup>d</sup>	148.2	155.3	55.6 <sup>c</sup>	168.4	189.7 <sup>b</sup>	53.4 <sup>c</sup>
12h	11.0	115.5 <sup>b</sup>	119.0	0.59 <sup>b</sup>	148.6	155.2	44.1 <sup>d</sup>	169.9	189.8 <sup>b</sup>	41.6 <sup>d</sup>

T<sub>g\_ons</sub>: T<sub>g</sub> at onset point; T<sub>g\_mid</sub>: T<sub>g</sub> at mid  $\Delta C_p$ ;  $\Delta C_p$  at glass transition; T<sub>c\_ons</sub>: initial temperature of re-crystallisation; T<sub>c\_peak</sub>: max temperature of the crystallization peak;  $\Delta H_c$ : enthalpy of crystallization; T<sub>m\_ons</sub>: initial temperature of melting; T<sub>m\_peak</sub>: max temperature of the melting peak;  $\Delta H_m$ : enthalpy of melting. <sup>a-f</sup>: different letters means significant difference ( $p < 0.05$ ) of the means in the same column

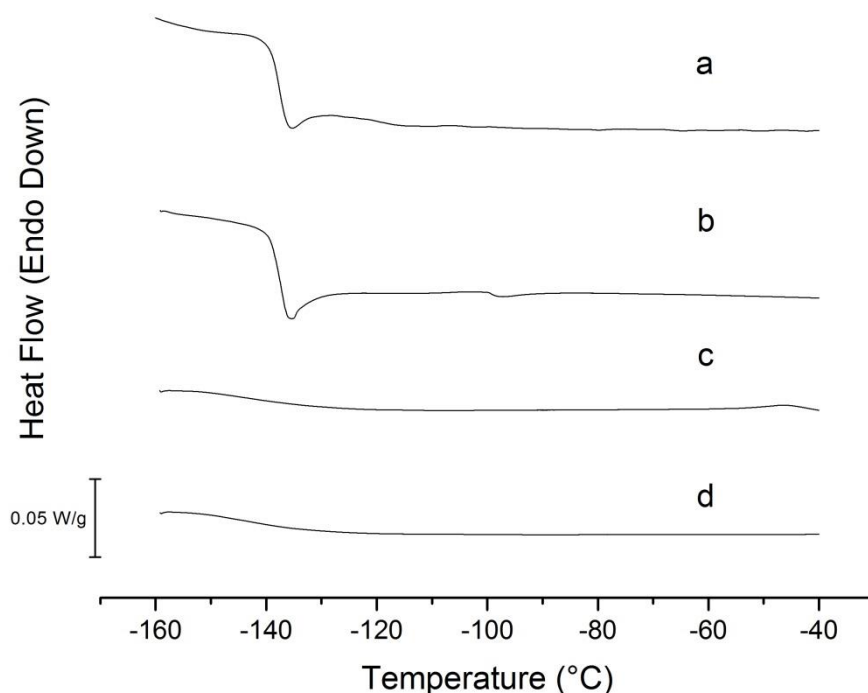
**Table 3.2.6.** Thermal data obtained from DSC scans of 12 h milled TRE mixed with increasing aroma concentration.

By mixing, (R)-Limo does not affect the kinetics of the cold-crystallization and of the melting of the crystalline fraction. On the contrary, the added limonene resulted to affect the entity of the state transitions by increasing them at low concentration (2.7%) while further higher aroma added in the mix caused a progressive decrease of the enthalpies of both crystallization and melting by keeping constant their relative ratio ( $\Delta H_m/\Delta H_c \approx 0,95$ ).

Glass transition is not significantly affected by the presence of limonene that may likely act as a dispersant, under the conditions of preparation of the sample. Since a dispersant is usually a (super)plasticizer with surface active properties that improve the separation of (micro)particles preventing their clustering. Therefore, it should be expected that limonene may both distribute at the surface of sugar particles and “dissolve” in the core of particles. This complex phenomenon is phenomenologically shown by the non-linear change of the  $\Delta C_p$  upon increasing limonene concentration: a decrease of its value (low amorphization of TRE) in the samples prepared with concentration of the aroma up to 5.2 %, is followed by an increase in the sample containing aroma at 11 %.

The different effect of the dispersion/solubilization state of limonene in the amorphous TRE matrix could be highlighted also by investigating the thermal properties of differently milled and comilled TRE+ (R)-Limo samples by evaluating the thermal behavior at low temperature ( $< 20^\circ\text{C}$ ).

Thermal analysis of pure (R)-Limonene evidence a main  $\Delta C_p$  jump characteristic of a glass transition located at  $-137.6$  (Table 3.2.7). No previous data on  $T_g$  of limonene were found in the literature that, based on our experiments, presents a  $\Delta C_p$  step at  $T_g$  of  $0,630 \text{ J } ^\circ\text{C}^{-1} \text{ g}^{-1}$ . The thermal scan of m-TRE (18 h) on the contrary did not show in the same temperature range thermal events (data not shown).



**Figure 3.2.16.** Thermograms ( $T$  range:  $-170^{\circ}\text{C}$  -  $-30^{\circ}\text{C}$ ) of pure (R)-Limonene (a), comilled TRE\_(R)Limo (18 h, 2.7%) mixed with 40 % of pure limonene (b); co-m TRE\_(R)-Limo (18 h, 2.7 %) (c); co-m TRE\_(R)-Limo (18 h, 11.0 %)

sample	Glass transition		
	$T_{\text{ons}}$ ( $^{\circ}\text{C}$ )	$T_{\text{mid}}$ ( $^{\circ}\text{C}$ )	$\Delta\text{Cp}$ ( $\text{J g}^{-1} \text{ }^{\circ}\text{C}^{-1}$ )
(R)-Limonene (pure)	-139.2	-137.6	0.63
co-m TRE_(R)-Limo (18h) (2.7 %)	-147.3	-142.7	0.12
co-m TRE_(R)-Limo (18h) (11.0 %)	-149.1	-142.9	0.14
co-m TRE_(R)-Limo (18h) (2.7 %) + 40 % (R)-limo (mixed)	-139.1	-137.4	0.26

$T_{g_{\text{ons}}}$ :  $T_g$  at onset point;  $T_{g_{\text{mid}}}$   $T_g$  at mid point

**Table 3.2.7.** Transition temperatures and  $\Delta\text{Cp}$  at glass transition pure (R)-Limonene and of comilled TRE\_(R)Limo with different aroma concentrations and 18 h-comilled TRE\_(R)Limo (2.7 %) mixed with 40%w/w of aroma

When comilled TRE\_(R)-Limo samples were analyzed under the same thermal conditions a similar event starting at lower temperature more broad and of lower intensity was detected. In both the 18 h-comilled samples prepared with two different initial aroma concentrations (2.7 % and 11.0 %) this thermal phenomenon was observed at a lower temperature than that observed for the pure limonene. The two samples showed the event at the same temperature but with a different  $\Delta C_p$ , higher in the case of the sample made of the higher concentration of limonene. This result could be related to the presence of limonene, its mobility in the matrix and its ability to undergo to a glass transition in the undercooled system. The addition of extra aroma (40 %w/w) by mixing to the 18 h-comilled TRE\_(R)-Limo 2.7% determined again the appearance of a glass transition in the range of temperatures of the pure limonene. No further experiments have been carried out on the differently milled/comilled trehalose and limonene as well as their mix. However these preliminary results allow us to give some insight to the dispersion state of limonene in the comilled glassy matrix of trehalose. Further experiments are needed to define the thermal behavior of limonene at temperatures below its melting point to better characterize the dispersion state in the solid, glassy trehalose matrix.

### **3.2.6. Effect of aroma enantiomeric form : (R)- versus (S)-Limonene**

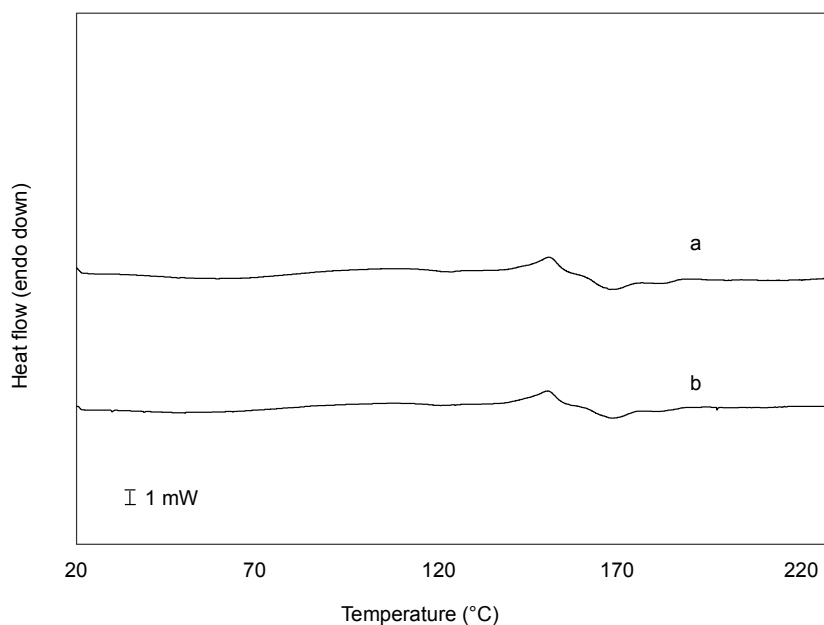
In nature limonene exists in two enantiomeric forms (R)- and (S)- characterized by slightly different chemical properties as well as sensory properties. In particular it is known that the S- enantiomer has a lower solubility in water than the R- one that seem to be referred to a more apolar, hydrophobic compound.

Comilling experiments have been carried out by adding 2.7% of the S-limonene in the initial mix and subjected to similar comilling conditions (12 h).

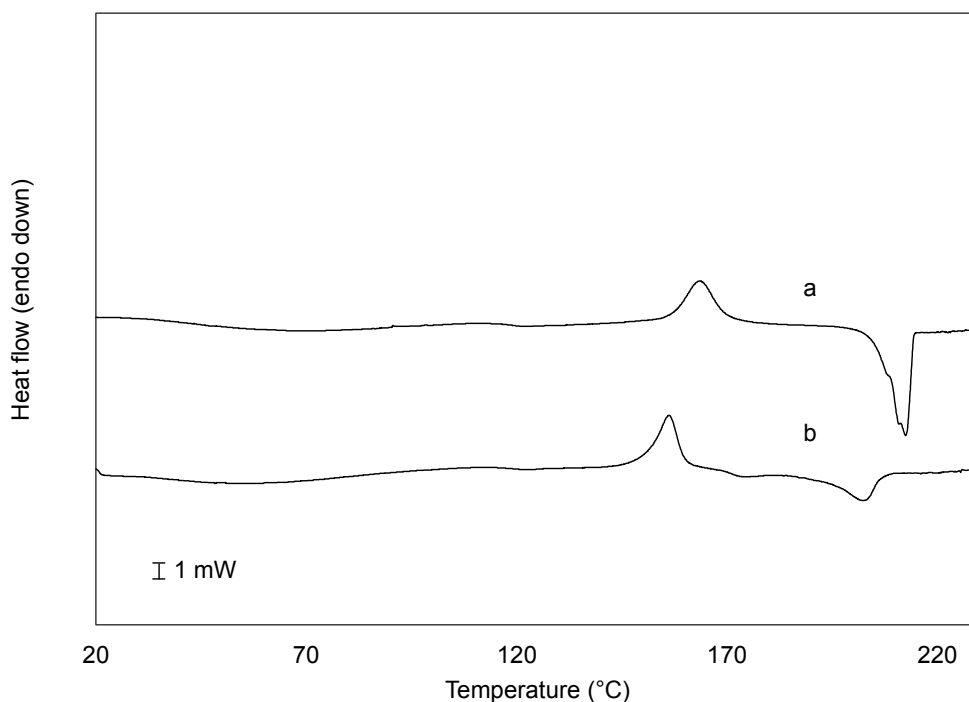
The addition of the (S)-limonene significantly affected the thermal behavior of the comilled trehalose (**Figure 3.2.17.**, **Figure 3.2.18.**, **Table 3.2.8.**). In particular, in the 6 h-comilled samples the presence of the more apolar enantiomer caused a shift of the  $T_{c_{peak}}$  by increasing it to a value similar to the sample without aroma along with a decrease of both the enthalpies of re-crystallization and melting. A similar effect is

observed also in the 12 h-comilled TRE\_Limo samples. Both at 6 and 12 h, however the comilled samples with the S-enantiomer showed  $\Delta H_c$  and  $\Delta H_m$  higher than those of the corresponding milled one. These results can be due to different effects of the S-Limo that influence the evolution of the crystal-to-glass transition during the process reducing or limiting the formation of the glassy matrix. Alternatively, the presence of the aroma in a dispersion state may influence the kinetics of the processes induced by the thermal conditions applied above the glass transition.

To this regards a significant difference of the retention of the aroma after the comilling process at both 6 and 12 h, has been observed between the powders made of (R)- and (S)- limonene (**Table 3.2.9.**), being for the latter significantly higher than that determined in the former. Moreover, SEM analysis allowed to highlight that com-TRE\_(S)-Limo are characterized by a microstructure more dense and compact in the larger granules to whom other granules of smaller size but less sharp is evidenced (**Figure 3.2.18.**).



**Figure 3.2.17:** Thermograms of co-m TRE\_Limo (6 h): effect of enantiomeric form: R- (a) vs S- (b)



**Figure 3.2.17:** Thermograms of co-m TRE\_Limo (12 h): effect of enantiomeric form: R- (a) vs S- (b)

Sample	Time (h)	Glass transition			1 <sup>st</sup> peak (exotherm, crystallization)			2 <sup>nd</sup> peak (endotherm, melting)			
		Tg <sub>ons</sub> (°C)	Tg <sub>mid</sub> (°C)	ΔCp (J g <sup>-1</sup> °C <sup>-1</sup> )	Tc <sub>ons</sub> (°C)	Tc <sub>peak</sub> (°C)	ΔH <sub>c</sub> (J g <sup>-1</sup> )	Tm <sub>ons</sub> (°C)	Tm <sub>peak</sub> (°C)	ΔH <sub>m</sub> (J g <sup>-1</sup> )	
m-TRE	6	0	113.5 <sup>a</sup>	120.1 <sup>a</sup>	0.37 <sup>d</sup>	137.1 <sup>d</sup>	148.5 <sup>b</sup>	38.5 <sup>d</sup>	159.1 <sup>c</sup>	166.4 <sup>c</sup>	34.3 <sup>f</sup>
Co-m TRE_(R)Limo		(R)-2.7 %	109.5 <sup>b</sup>	118.6 <sup>b</sup>	0.43 <sup>c</sup>	138.6 <sup>d</sup>	144.3 <sup>d</sup>	58.0 <sup>c</sup>	153.9 <sup>d</sup>	165.4 <sup>c</sup>	75.1 <sup>d</sup>
		(S)-2.7 %	111.0 <sup>b</sup>	119.5 <sup>b</sup>	0.58 <sup>b</sup>	137.2 <sup>d</sup>	147.6 <sup>d</sup>	42.1 <sup>c</sup>	156.6 <sup>d</sup>	165.5 <sup>c</sup>	47.6 <sup>d</sup>
m-Treha	12	0	110.9 <sup>b</sup>	117.9 <sup>c</sup>	0.65 <sup>a</sup>	148.7 <sup>c</sup>	155.3 <sup>c</sup>	68.9 <sup>b</sup>	168.7 <sup>b</sup>	175.4 <sup>b</sup>	62.9 <sup>d</sup>
Co-m TRE_(R)Limo		(R)-2.7 %	109.1 <sup>b</sup>	118.9 <sup>b</sup>	0.59 <sup>b</sup>	149.4 <sup>bc</sup>	154.2 <sup>c</sup>	71.3 <sup>b</sup>	199.2 <sup>a</sup>	206.9 <sup>a</sup> <sub>b</sub>	86.3 <sup>b</sup>
		(S)-2.7 %	111.9 <sup>b</sup>	118.2 <sup>b</sup>	0.63 <sup>a</sup>	150.1 <sup>d</sup>	155.0 <sup>d</sup>	65.9 <sup>c</sup>	168.4 <sup>d</sup>	196.5 <sup>c</sup>	72.3 <sup>d</sup>

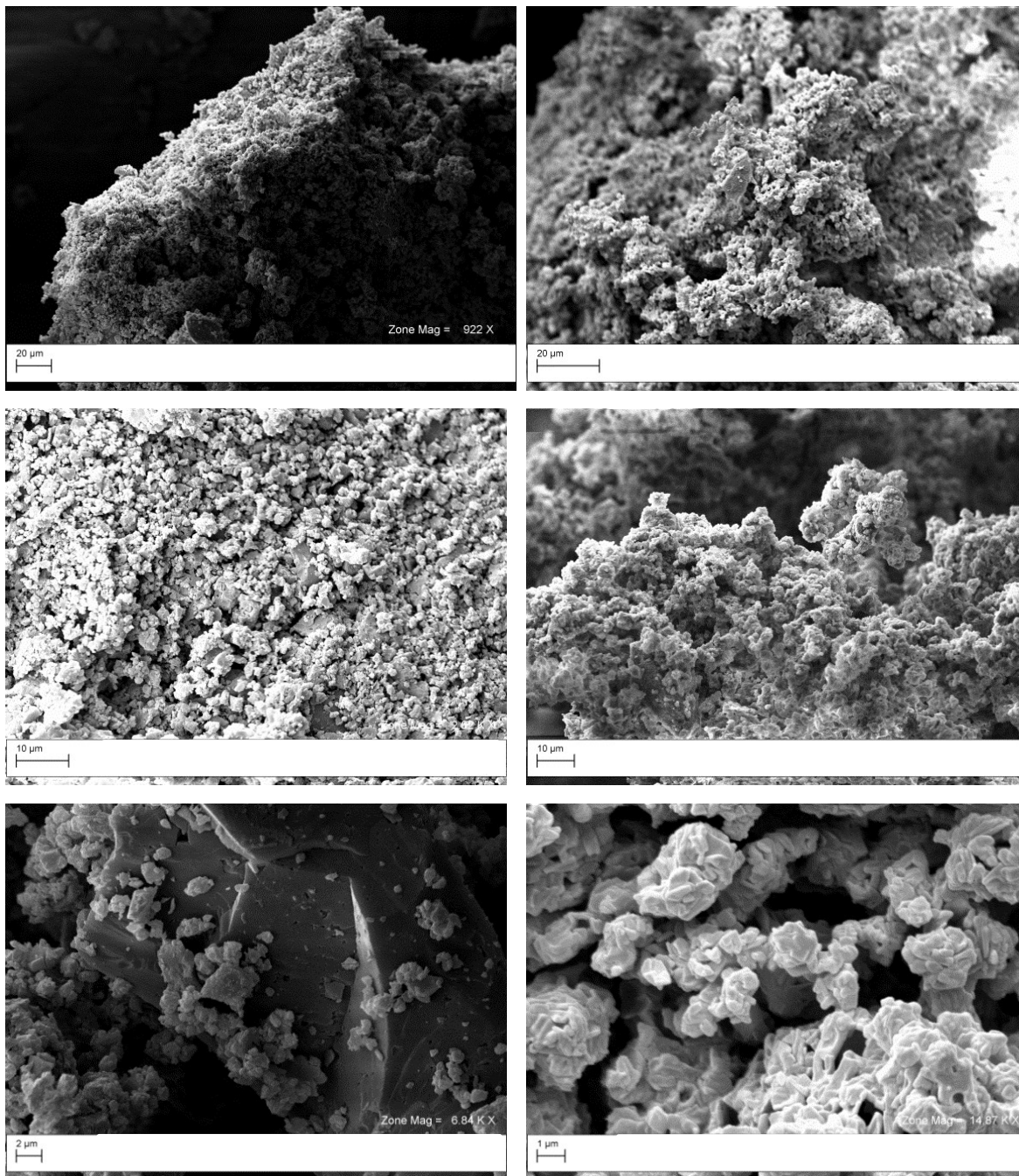
Tg<sub>ons</sub>: Tg at onset point; Tg<sub>mid</sub>: Tg at mid ΔCp; ΔCp at glass transition; Tc<sub>ons</sub>: initial temperature of re-crystallisation; Tc<sub>peak</sub>: max temperature of the crystallization peak; ΔH<sub>c</sub>: enthalpy of crystallization; Tm<sub>ons</sub>: initial temperature of melting; Tm<sub>peak</sub>: max temperature of the melting peak; ΔH<sub>m</sub>: enthalpy of melting. <sup>a-f</sup>: different letters means significant difference (p<0.05) of the means in the same column

**Table 3.2.8.** Thermal data obtained from DSC scans of milled TRE and comilled TRE\_(R)-Limo and comilled TRE\_(S)-Limo for 6 and 12h..

Limonene enantiomer	Milling	
	time (h)	Retention (%)
R-	6	61.4
	12	76.8
S-	6	76.5
	12	84.6

**Table 3.2.9.:** Retention of (R)- and (S)-limonene in comilled TRE\_Limo amorphous powders after 6 and 12 h processing time.

NMR results allowed to determine limited differences between the comilled samples prepared with the two different enantiomers. The T2 average parameter that includes the contributions of both the solid and liquid component resulted significantly affected by the different retained limonene in the matrix being higher in the com-TRE\_(S)-limo sample than in the other. On the contrary, values of the M2 parameter resulted slightly lower than those of the samples made with the R- enantiomer but with no statistical difference between them ( $7974 \text{ s}^{-1}$   $7898 \text{ s}^{-1}$ , R- and S- comilled samples respectively). This may be an evidence a morphology of a glassy matrix with weak interactions and less rigidity, due to the higher concentration of the aroma differently distributed and dispersed within the system.



**co-m TRE\_(R)-Limo (2.7 %, 12h)**

**co-m TRE\_(S)-Limo (2.7 %, 12h)**

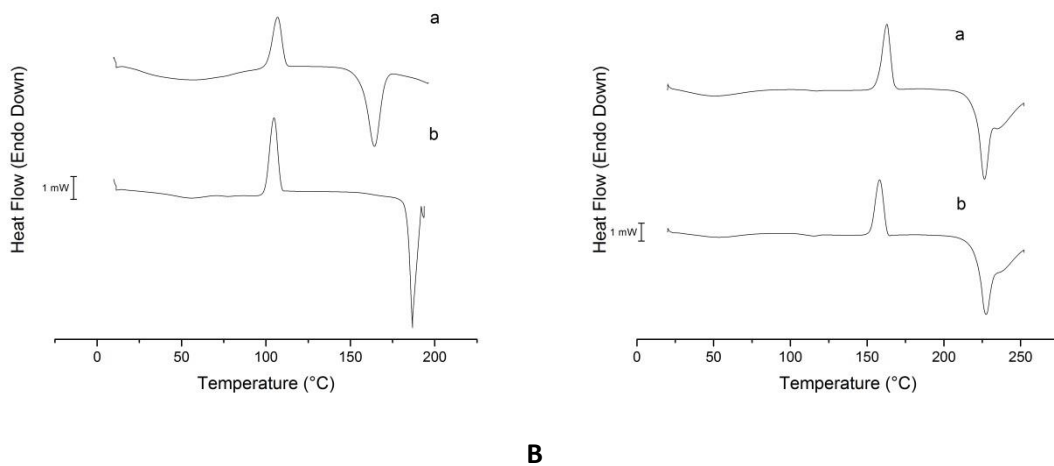
**Figure 3.2.18.** Morphology of comilled TRE\_(R)Limo (left images) comilled TRE\_(S)Limo (2.7% obtained after 12 h processing time as observed by SEM. Magnification for each image is given on each SEM image.



### 2.1.1. Sugar type

Milling of TRE and comilling of TRE in presence as cosolute of limonene have been shown to induce mechanically a crystal-to-glass transition of the saccharide along with a significant aroma encapsulation/retention effect.

To investigate further the exploitation of this solid state amorphization technique on different matrices than trehalose, as well as to highlight differences in the results obtained with trehalose in respect to other disaccharides, experiments have been also carried out by applying milling/comilling with the same aroma ((R)-limonene) on two different disaccharides, and in particular, sucrose and lactose. Process and formulation conditions were chosen based on their more wide investigation in this study and applied (2.7% aroma, 12 h process time).



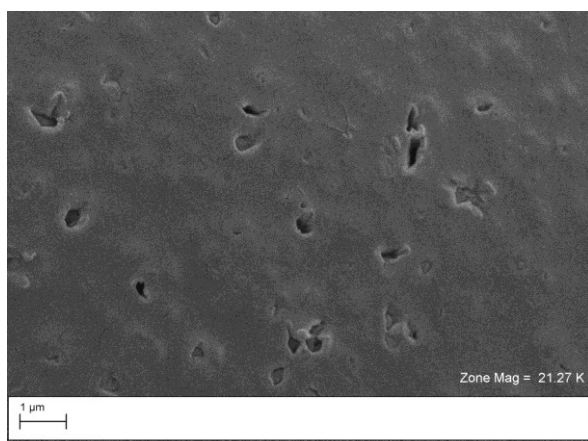
**Figure 3.2.20.:** Thermograms of sucrose (A) and b-lactose (B) after milling and comilling (12 h) with (R)-limonene (2.7 %w/w). (a) milled (b) comilled samples.

sample	(R)-Limo (%)	Glass transition			1 <sup>st</sup> peak (exotherm, crystallization)			2 <sup>nd</sup> peak (endotherm, melting)		
		Tg <sub>ons</sub> (°C)	Tg <sub>mid</sub> (°C)	ΔCp (J g <sup>-1</sup> °C <sup>-1</sup> )	Tg <sub>ons</sub> (°C)	Tg <sub>mid</sub> (°C)	ΔCp (J g <sup>-1</sup> °C <sup>-1</sup> )	Tg <sub>ons</sub> (°C)	Tg <sub>mid</sub> (°C)	ΔCp (J g <sup>-1</sup> °C <sup>-1</sup> )
Milled sucrose	0	72.1	73.5	0.62 <sup>a</sup>	98.9 <sup>b</sup>	106.8 <sup>a</sup>	53.3 <sup>b</sup>	148.7 <sup>b</sup>	164.2 <sup>b</sup>	107.7 <sup>a</sup>
Comilled sucrose_(R)Limo	2.7	71.1	73.4	0.21 <sup>b</sup>	96.7 <sup>a</sup>	104.7 <sup>b</sup>	61.3 <sup>a</sup>	181.5 <sup>a</sup>	186.8 <sup>a</sup>	63.9 <sup>b</sup>
milled β-lactose	0	110.1	114.0	0.56	156.7 <sup>a</sup>	162.6 <sup>a</sup>	82.2 <sup>a</sup>	219.9	226.6	249.0 <sup>b</sup>
comilled β-Lactose_(R)Limo	2.7	113.8	113.8	0.55	152.8 <sup>b</sup>	158.2 <sup>b</sup>	75.3 <sup>b</sup>	220.4	227.9	295.5 <sup>a</sup>

Tg<sub>ons</sub>: Tg at onset point; Tg<sub>mid</sub>: Tg at mid ΔCp; ΔC<sub>p</sub> at glass transition; Tc<sub>ons</sub>: initial temperature of re-crystallization; Tc<sub>peak</sub>: max temperature of the crystallization peak; ΔH<sub>c</sub>: enthalpy of crystallization; Tm<sub>ons</sub>: initial temperature of melting; Tm<sub>peak</sub>: max temperature of the melting peak; ΔH<sub>m</sub>: enthalpy of melting.

<sup>a-b</sup>: different letters means significant difference of the means of milled and comilled samples of each disaccharide.

**Table 3.2.9.** Thermal data obtained from DSC scans of milled sucrose and β-Lactose and corresponding comilled\_(R)-Limo (2.7 %) for 12 h.



**Figure 3.2.21:** Image of co-milled sucrose (R)-Limo (2.7%) obtained by Scanning Electron Microscopy

Milled sucrose presents a Tg at 73.5±0.2 that is followed by an exothermal peak associated to the re-crystallization of the amorphous sugar formed during milling starting at ca. 99°C and by an endothermal one related to the melting of the crystalline fraction starting at 148°C. Tg values are in agreement with that of Megarry et al. (2014) on milled sucrose obtained by applying slightly different process conditions (cryomilling) and slightly higher than that of Wildfong et al (2006) (71.5 ±0.9). Megarry

et al. (2014) observed the start of the cold crystallization at a lower temperature (93.5 °C) but a similar melting onset (ca. 146 °C).

The thermal behavior of the initial crystalline sucrose were not tested. However, literature data on a similar commercial product (Sigma) reports an onset melting temperature at  $158.9 \pm 0.2$  °C (Megarry et al., 2014) and a  $\Delta H_m$  (at  $10$  °C  $\text{min}^{-1}$ ) of  $126.4 \text{ J g}^{-1}$ . As an index of the extent of amorphization induced by the milling, the  $\Delta H_m / \Delta H_c$  ratio was used also in this case and a value of ca. 1 was obtained. On the other side,  $\Delta H_c$  resulted lower than that of the crystalline product indicating that not all the saccharide underwent to amorphization upon ball milling.

Upon comilling in presence of (R)-Limonene, while  $T_g$  didn't change, its  $\Delta C_p$  resulted significantly lower. Moreover the melting onset and its enthalpy resulted respectively higher and lower than that of the sucrose milled for the same time.

Limonene seems not to act as plasticizer in the sugar affecting its glass transition. However, when the rubbery state, the aroma increases the entity of the re-crystallization, likely by increasing the mobility of the system where limonene results entrapped (Figure 3.2.21). A limonene retention equal to  $55.6 \pm 3.2$  % almost all entrapped/encapsulated in the glassy sucrose matrix ( $< 0.3$  % at surface).

Commercial  $\beta$ -lactose was used without preliminary modifications to reduce the content of the  $\alpha$ -lactose anomer whose presence is due to the process used by the manufacturing company. Also in this case milling determined significant changes in the thermal behavior of the corresponding initial disaccharide that showed only a main and broad melting event at a temperature above 220 °C. No further experiments and analysis have been made to determine the amorphization degree of this disaccharide but some authors indicate that after 2 h of milling carried out under the same process conditions applied for this experiment are sufficient to determine a full amorphisation of lactose (Caron et al., 2011; Descamps et al., 2006). The analysis of the thermograms of the milled sample evidences the glass transition event at a temperature in agreement with literature data, along with the appearance of the exothermic peak of cold crystallization and the endothermic one associated to the melting.

The presence of a fraction of  $\alpha$ -anomer in the sample should evidence some difference in the phenomenology of the crystalline phase, either if two crystalline phases are present or if the two anomers co-crystallize. Indeed, the shape and trend of the melting peak show evidence of a shoulder at higher temperatures.

In this case, the co-presence of the liquid aroma during the high energy milling process as shown for sucrose made easy the re-crystallization process while it reduced its entity. However, a higher  $\Delta H_m$  was observed than that of the corresponding milled sample. Overall these results could correspond to a lower amorphization of the sugar induced by milling while its presence in the amorphous matrix resulted to reduce the kinetics of cold-crystallization.

The comilled  $\beta$ -lactose retained  $60.4 \pm 1.3$  % of (R)-Limonene in the amorphous matrix, with a higher efficiency than that observed for the sucrose.

The effect of the different chemical and physical properties of the disaccharides and their corresponding ability to encapsulate/retain aroma compounds needs to be further investigated.

### 3. PHYSICAL STABILITY OF AMORPHOUS FLAVOR ENCAPSULATING MATRICES AND AROMA LOSS

A glassy state could be considered as a “solid solution” with a rather high viscosity (ca.  $10^{12}$  Pa) formed at non-equilibrium conditions either by desolvation of the dispersing medium (water) or from a melt by cooling and quenching (Bhandari and Howes, 1999). Mechanical stresses like those determined by milling has been shown able to determine the formation of glasses from crystalline states (Willart and Descamps, 2008). Glassy, amorphous states are metastable systems not in thermodynamic equilibrium and therefore tend to reach its lowest free energy by crystallization processes (Slade et al., 1993, Flink, 1983). Despite the high viscosity, water mobility has been shown to occur in glassy food by several instrumental investigations (see reviews Roudaut et al., 2004 and Le Meste et al., 2002).

In glassy systems volatile compounds are generally retained according to the entrapment and “diffusion limited” mechanisms and their loss occurs only via the pores of the matrix. This implies an effect of the microstructural properties of the glassy matrix and the role of the process used to obtain the glassy matrix, scarcely studied.

The stability of the amorphous systems and the glass-to-crystal transformation over time has been shown to be a function of  $T-T_g$  (where T refers to the storage temperature and  $T_g$  to the glass transition temperature of the specific system) (Senoussi et al., 1995, Roos and Karel 1991b). The increase of the temperature or of the water content at conditions that bring the glassy material to a rubbery state can promote aroma release. Levi and Karel (1995) have described the release of propanol from freeze-dried sucrose and sucrose-raffinose matrices with a kinetic depending on  $T-T_g$ , in a range of  $(T-T_g)$  approximately 10 to 30 °C that could be modelled according to the WLF kinetics with the “universal” coefficients (Levi and Karel 1995).

Moreover, aroma release seems to be highly dependent also on structural changes occurring in the glassy matrix induced by the increased mobility around and above  $T_g$ . These include crystallization, collapse and caking phenomena.

Crystallization process, by increasing the intermolecular interactions between the carbohydrate molecules and thus, by decreasing the “free” space where the aroma is entrapped, has been affecting the retention of aroma compounds like diacetyl and 1-n-propanol in lactose and sucrose based glassy matrices (Senoussi et al., 1995; Levi and Karel, 1995).

If amorphous metastable systems are kept below their  $T_g$ , crystallization phenomena may occur locally by residual solvent (water) migration that may induce the formation of nano-crystallites.

On the contrary, caking and collapse phenomena are associated to a reduced volatile loss, due to the formation of less permeable and porous layers allowing the matrix-to-air transfer (Le Meste et al., 2002).

In our study differently amorphized low-moisture TRE systems prepared with or without (R)-limonene have been stored for up to 6 months under low relative humidity (< 10 % RH) and controlled ambient temperature (20 °C) to evaluate the effect of the encapsulated aroma compound on the physical properties of the glassy matrices and the corresponding retention of the volatile.

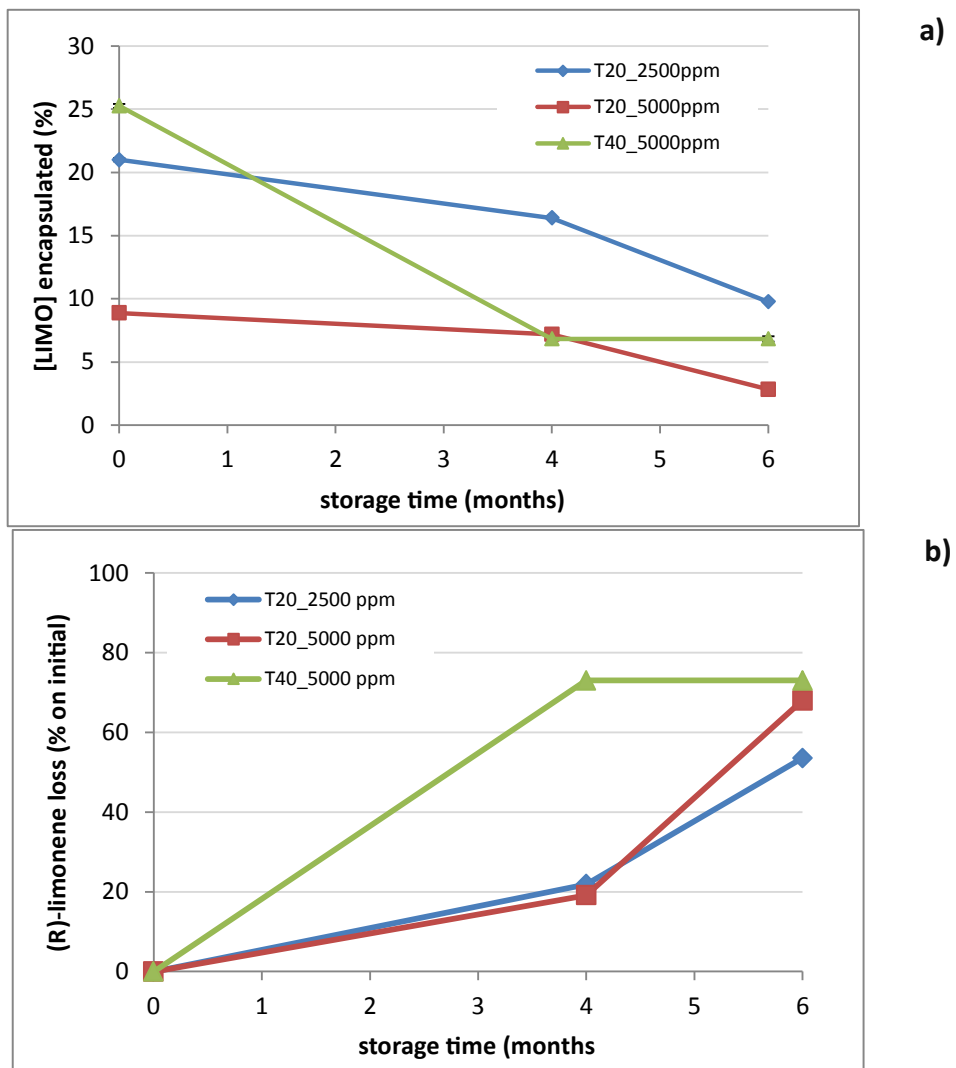
Under these experimental conditions and the results of the thermal analysis evaluation it is supposed that physical changes of the amorphous matrices could occur only due to the residual moisture and the presence of encapsulated/entrapped limonene.

In **Figure 4.1.a.** the variation of the (R)-limonene content in the spray dried matrices differently processed and made from different TRE and aroma concentrations up to 6 months of storage. To allow a better comparison of the changes occurring over time, the loss of the aroma expressed on the initial content of the same samples just after their preparation is reported in **Figure 4.1.b.**

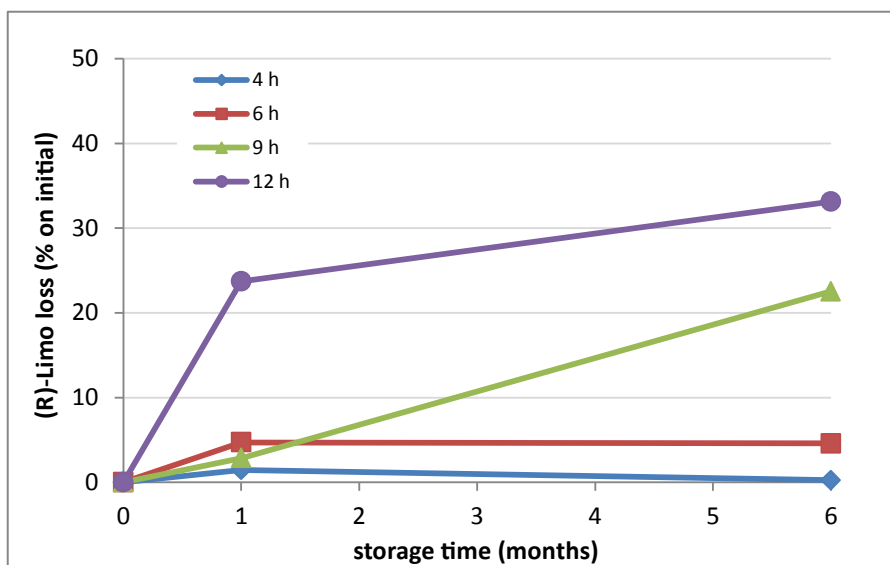
Similar data of limonene loss in respect to that determined in the just prepared samples for the co-m TRE\_(R)-Limo samples at different comilling time (4, 6, 9 and 12 h) is reported in **Figure 4.2** as a function of storage time.

It has to be pointed out that in the 6 months-stored glassy matrix no presence of limonene derivatives have been detected in the chromatogramme.

By the comparison of the results obtained after 6 months in the two series of samples, the results evidence a main and significant effect of the amorphization procedure of TRE in presence of limonene (i.e. spray drying and comilling) on the ability to retain the encapsulated aroma under investigation that have significantly affected both the residual moisture content and the microstructural properties.



**Figure 4.1.** Change of the (R)-Limonene content during storage time in spray dried powders obtained from TRE solutions (20%) and TRE\_(R)Limo microemulsions (20%, 2500 and 5000 ppm) (a) and the corresponding loss (%) computed in respect to the aroma content of the samples just after preparation (b).



**Figure 4.2.** Change of the (R)-Limonene loss (%) computed in respect to the aroma content of the samples just after preparation of comilled TRE\_(R)Limo (2.7% limonene) processed for 4, 6, 9 and 12 h as a function of storage time.

The trend of aroma loss as a function of storage time reported in Figure 4.1 for the spray-dried series sample highlight that after 6 months of storage over 50 % of the (R)-Limo encapsulated during processing is lost. Moreover, at that storage time the loss results higher for the samples prepared with the higher limonene concentration in the initial feeding dispersed system.

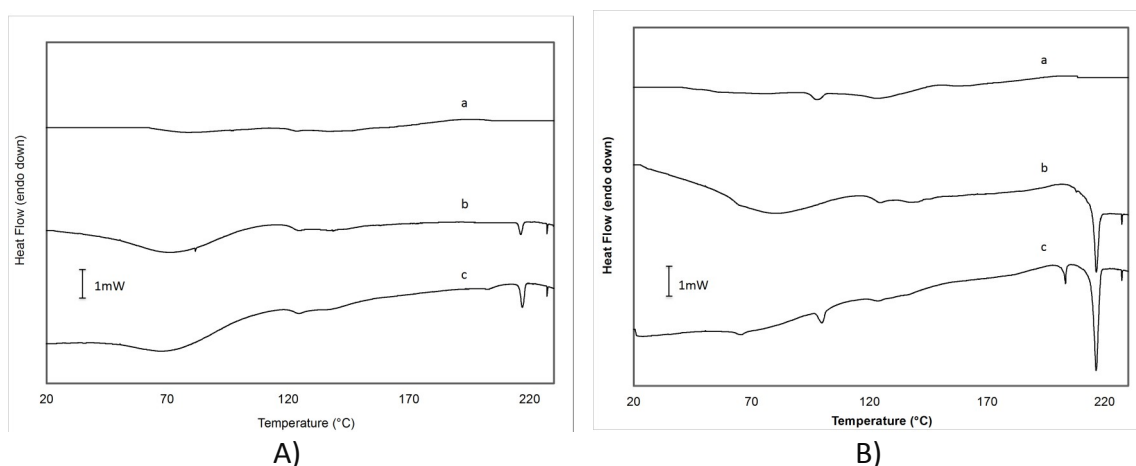
A number of papers deal with the retention of aroma compounds in spray dried saccharide systems and among them few are also focused on limonene (or limonene containing essential oils) retention (e.g. Liu et al., 2001; Sosa et al., 2014). All these studies and experiments are aimed to optimize carrier formulation and therefore, surface active compounds and/or film forming carbohydrates and hydrocolloids that are known to influence during the desolvation process in the spray drying chamber and the physical properties of the final low moisture amorphous powders, are included in the initial formulation. In general in these studies very high retention abilities have been observed both after processing and over storage time up to 100%.

On the contrary, our spray-dried samples do not contain additional surface active compounds and the loss may occur by diffusional mechanisms through the pores and voids structure of the amorphous matrices (Goubet et al., 1998).



For small molecules in a fully amorphous system, the permeability of the molecules has been related to the amorphous free volume (Karel et al., 1975). To this regards, no specific analysis have been performed (e.g. PALS).

However some changes in the glassy state have been observed by thermal analysis of the spray-dried powders after 6 months. A significant increased melting of the crystalline fraction has been observed in the thermograms reported in **Figure 4.3** of the powders obtained by desolvation of 20 % TRE solution and 20% TRE solution containing 2500 ppm of limonene. Based on the  $\Delta H_m$  of the crystalline fraction the increase of the crystalline fraction results different in the glassy dried powders differently processed. While no significant changes in the 6 months of storage were observed in the  $\Delta H_m$  of the powders obtained from 20 % TRE solutions the melting enthalpy value increased from 1.65 to 19.7 J g<sup>-1</sup> and from 4.2 to 29.3 J g<sup>-1</sup> for those obtained from 20% TRE+2500 ppm and 20% TRE +5000 ppm limonene respectively.



**Figure 4.3.** Thermograms of spray dried 20 % TRE solution (A) and 20 % TRE + 2500 ppm of (R)-Limo at different storage time. a)  $t=0$  – just after preparation; b) after 4 months; c) after 6 months.

Crystallization could have been favored by the dispersed/entrapped/encapsulated limonene; however, the residual water not removed from the sample during samples preparation could also have caused local mobility and crystal nucleation.

Other microstructural aspects like particle size and surface area of the powders of different entity depending on the process conditions and formulation could also be implied in the different entity of the volatile release and loss during storage.

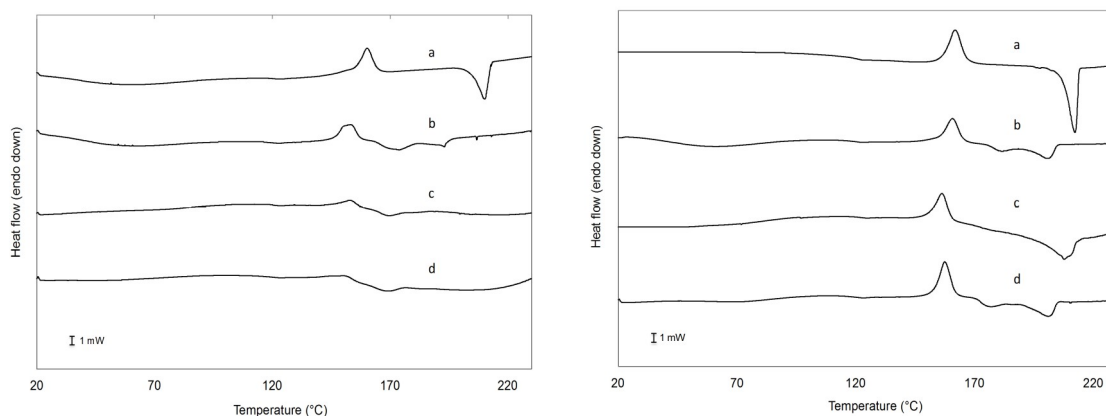
Comilled samples, contrary to those obtained by spray drying, presented a significantly higher volatile retention ability during storage time with a loss as high as 32 % determined in the sample series comilled for 12 h.

Comilled TRE\_Limo systems processed for lower time show a lower loss of limonene that progressively decreases up to be almost equal to zero for the sample comilled for 4 h. These data could be associated to the different amorphization degree of the initial samples, being lower for the ones undergone to the lower comilling time. The co-presence of a crystalline fraction in amorphous encapsulating matrices has been already recognized to exert better performances in retaining volatile compounds than fully amorphous matrices (Benoussi et al., 1995, ) that could have developed a mixed and complex amorphous-crystalline with better volatile retention properties.

Thermal analysis of the 6 months-stored samples has been carried out (see for example, the thermograms of some samples in **Figure 4.4**) with interesting results that here below are summarized:

- No significant changes in  $T_g$  values have been observed in all differently milled and comilled samples.
- 4 h-comilled TRE\_Limo samples do not present significant changes due to storage time in the thermal behavior.  $T_g$ , re-crystallization and melting of the crystalline fraction of the samples both in presence and absence (milled) of limonene did not show significant changes in respect to the samples at  $t=0$ . The co-presence of glassy and crystalline TRE not amorphized during process besides the very low mobility of the system well below its  $T_g$ , may have hindered any further physical change by reducing the mobility in the matrix.
- 6 h-, 9 h- and 12 h- milled TRE samples: some changes in the thermal behavior of the milled TRE above the  $T_g$  occur with a significant increase of the enthalpy of re-crystallization and a limited or no decrease of the corresponding  $\Delta H_c$ .
- 6 h-, 9 h- and 12 h- comilled TRE-Limo samples While the 6h- and 9 h co-milled samples do not change their thermal properties in respect to the initial samples, the 12 h-co milled sample showed a significant decrease of the  $\Delta H_m$  ( $t_0 = 71.9 \text{ J} \cdot \text{g}^{-1}$  vs  $t_{6m} = 60.7 \text{ J} \cdot \text{g}^{-1}$ ) along with an increase of the  $\Delta H_c$  ( $t_0 = 85.5 \text{ J}$

$\cdot g^{-1}$  vs  $t_{6m} = 123.1 J \cdot g^{-1}$ ). The higher presence of an amorphous fraction and the presence of the limonene entrapped and finely dispersed in submicron and nano-droplets may have acted as plasticizer by contributing to the mobility of the high viscous and glassy matrix of the more amorphized comilled system.



A)

B)

**Figure 4.4.** Thermograms of milled TRE and comilled TRE\_(R)Limo (2.7%) for 6 h (A) and 12 h (B) at  $t=0$  and after 6 months of storage. a) m-TRE  $t=0$ ; b) co-m TRE  $t=0$ ; c) m-TRE  $t=6$  months; d) co-m TRE  $t=6$  months.

Also for the comilled samples the loss of limonene could, thus, be associated to the crystallization phenomenon that caused the loss of the ability of the amorphous matrices by increasing the molecular interactions of the trehalose in the matrix. However, diffusional phenomena through the powder could have been taken place during the storage time.

## 5. CONCLUSIONS AND PERSPECTIVES

The research aim of this project focused on shedding light on the role of some physicochemical and structural features of the matrix that affect the retention/release of aroma volatile compounds in glassy matrices. The study has been carried out on trehalose and limonene (the R-enantiomer), as model carbohydrate and aroma compound, respectively.

The achievement of a glassy state is critical to encapsulate bioactives and flavor compounds that become entrapped in the system during the vitrification process. However, process conditions, thermal history and compositional values affect the retention ability and the physical properties of the low moisture/dry encapsulating amorphous materials. To this aim two glass-forming processes with a different thermal history and starting material morphology have been considered. In particular, the spray-drying applied to saccharide solutions is representative of the desolvation techniques, and milling of initially crystalline matrix is representative of solid-state amorphization techniques.

Despite the different thermal history, in both cases a considerable encapsulation effect was obtained with liquid limonene retained in the glassy, low moisture matrix.

The physical and structural analyses along with the evaluation of the system mobility (NMR) and of the limonene retention provide results that shed light on the mechanisms of aroma entrapment in glassy carbohydrate matrices.

In summary the key-points are:

- Micro- and nano-structure: despite the common powder-like characteristics, the two processes determine the formation and development of encapsulating glassy matrices with significantly different micro- and nano-structural and physical properties as evaluated by interpreting the calorimetric results. Concerning the micro-and nano-structure, different thermal history and energy input are implied during the process obtaining amorphous particles of different shape and size. Limonene did affect the size of the round-shape spray-dried particles. On the other side, in the co-milled samples, the formation of

submicron, nano-dispersions of limonene in the glassy trehalose has been evidenced. Due to the intrinsic metastability of an incompatible mixture, this outcome is relevant for the product stability upon storage. It is worth mentioning that this aspect is affected also by the residual moisture of the samples after preparation.

- Thermal properties: different thermal behavior is evident for the spray-dried and milled/comilled TRE+Limonene systems as affected by the processes applied. These findings are in agreement with the literature and previous studies carried out by Cesàro and co-workers on trehalose and Descamps and co-workers on milling and comilling. Presence and content of limonene in both spray-dried and comilled amorphous systems did not determine changes in the  $T_g$  of the glassy systems. On the contrary in the comilled matrices, depending on content and enantiomer (R- vs S-) significantly different kinetics of cold-crystallization in the rubbery state above  $T_g$  have been observed, with consequences on the melting.
- Limonene retention: the most important and novel result is that retention in the glassy matrix resulted significantly higher in the comilled processed system. Thermal history of the system during amorphization is an important factor affecting the retention of volatile compounds due to their intrinsic volatility. It should be stressed the absence, in our systems, of any additional film forming molecules or surfactants able to contribute to the formation of the coated droplets during spray drying. On the contrary, comilling process carried out at room temperature does not induce limonene liquid-vapor transition. The enhanced surface properties and the liquid-vapor partition of limonene in trehalose solutions at increasing solute concentration could also be taken as enhancement of the aroma loss during process. In conclusion, micro- and nano-structural characteristics as well as presence of residual nano-crystals in the differently obtained glassy matrices can be at the basis of the different retention values during storage.

- NMR analysis highlighted a different molecular mobility and strength of interactions in the glassy matrices as a consequence of the process conditions and presence and concentration of the limonene.

Overall the results of this study provide the hypothesis that limonene, a relatively high hydrophobic compound, is encapsulated in the differently amorphized glassy, trehalose matrices as discrete sub-micron and nano-droplets as in a liquid-solid dispersion. The nano-dispersed limonene seems to be confined in the “free” volume of the matrix without modifying the main strength of the molecular interactions of the saccharide. In the comilled systems limited amounts of limonene could also be partly adsorbed onto the surface of glassy and of the crystalline, not amorphized particles.

Results of this project will be of interest not only for the development of new flavor-encapsulated products and ingredients, but also for selecting the most appropriate processing technique. The potential of milling and comilling techniques has been investigated and the results show the evidence for future commercial exploitation.

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## 8. PAPERS

Results reported in this thesis are unpublished data even if presented at some international conferences and symposium by publication only of the abstract on the corresponding book of abstracts. Oral and poster presentations have been delivered at the following international events:

- 29<sup>th</sup> EFFoST conference, 10-12 November 2015, Athens (GR) (oral presentation: Pittia P. et al. *Physical and microstructural properties of amorphous co-milled sugars and aroma*).
- Delivery of Functionality in Complex Food Systems (VI) 14-17 July 2015, Paris (FR) (poster: Pittia et al., *Encapsulation of aroma compounds in amorphous sugars systems by co-milling*).
- Amorph 2014, The Felix Franks Symposium Cambridge (UK) 14-16 July 2014 (oral presentation: Pittia et al., *Development of carbohydrate amorphous matrices as flavor carriers*).
- Delivery of Functionality in Complex Food Systems (V) 30 Sept 2013, Haifa (IL) (oral presentation: Pittia et al., *Flavour retention in carbohydrate amorphous matrices*).

The following manuscripts are under preparation for submission to peer-reviewed international journals:

- Pittia P., Faieta M., Cesàro A. Physical and microstructural properties of amorphous co-milled sugars and aroma. *Invited paper to be submitted to Innovative Food Science and Emerging Technology*  
Pittia P., et al. Process parameters and formulation affecting amorphization of trehalose and encapsulation of limonene by comilling
- Pittia P., et al., Effect of glass-forming processes on thermal and structural properties of trehalose and limonene amorphous matrices
- Pittia P., et al., Interfacial and colloidal properties of limonene/sugar microemulsions.