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CHARACTERIZATION OF CARBOHYDRATES AS FOOD QUALITY, PROCESS AND TIPICITY MARKERS BY HPAEC-PAD OPTIMIZED AND VALIDATED METHODS

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Preface

Carbohydrates are one of the most important macronutrients in many foods. Individual molecules could be classified according to the number of monomers that they contain as monosaccharides, oligosaccharides or polysaccharides. It is important to determine the type and concentration of carbohydrates in foods for many reasons like:

- *Standards of Identity* - foods must have compositions which conform to government regulations
- *Nutritional Labeling* - to inform consumers of the nutritional content of foods
- *Detection of Adulteration* - each food type has a carbohydrate "fingerprint"
- *Food Quality* - physicochemical properties of foods such as sweetness, appearance, stability and texture depend on the type and concentration of carbohydrates present.
- *Economic* - industry doesn't want to give away expensive ingredients
- *Food Processing* - the efficiency of many food processing operations depends on the type and concentration of carbohydrates that are present

In the first part of PhD thesis, both simple and complex carbohydrates are studied in many foods like leguminosae, pasta, honey , proposing carbohydrates as markers of food process, quality and authenticity.

Our attention is then focused , in the second part, on fructans analyses, both in natural sources of these soluble fibers (e.g. onions and shallots) and in functional foods (e.g. fermented milks and cooked ham) where fructans are added for their prebiotics properties and for their lower calorie intake than simple sugars.

In particular the thesis was structured in a general introduction regarding the analytical methods and the type of single and complex carbohydrates in foods and in chapters subdivided according the food analyzed like:

- **leguminosae** (Chapter 3):: proposal of a new sugary substrate for *Saccharomyces cerevisiae sp.* metabolism
- **pasta** (Chapter 4): determination of reducing sugars proposing maltulose as quality marker of pasta drying process in relationship with furosine and colour analysis
- **prebiotic and synbiotic fermented milks** (Chapter 7): characterization and quantitation of fructooligosaccharides and inulooligosaccharides in relationship to probiotics metabolism
- **onions** (Cchapter 8): discrimination of onions of different cultivar and geographical origins by chromatographic profiles fingerprinting and chemometric analysis

Preface

High performance anion exchange chromatography (HPAEC) with pulsed amperometric detector (PAD) was demonstrated to be a very useful, selective and sensible tools for all carbohydrates analyzed. All methods were optimized in terms of the choice of column and the gradient elution in relationship with the degree polymerization (DP) of carbohydrates.

A new quantitative method was proposed for fructans analysis which commercial standards are not available and compared with Official AOAC method (Chapter 6). Furthermore mass spectrometry (MS) analysis (Chapter 5) were conducted with the aim to establish the correct assignment of the degree of polymerization to fructans and to foods which no notice about carbohydrates composition are reported in labels.

Finally fructans food safety was tested in terms of nitrate content by capillary zone electrophoresis (CZE) (Chapter 9).

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1. PREBIOTICS

1.1 Definition of prebiotics

The definition of prebiotics was updated in 2004 and prebiotics are now defined as ‘‘selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health”(Gibson et al, 2004).
This concept implies that some dietary components resist the hydrolysis by the digestive enzymes and/or are not absorbed in the upper part of the gastro-intestinal tract including the small intestine. Indeed, these compounds must pass into the large bowel where most of the indigenous intestinal microbiota are located (Roberfroid, 2002).

1.1.2 Type of prebiotics

A range of substrates of dietary origin, or produced by the host, are available for fermentation by the colonic microflora. Through diet, resistant starch (RS) is the most quantitatively important. Non-starch polysaccharides (NSP) form the next largest contribution and include plant-derived substrates such as pectin, cellulose, hemicellulose, guar and xylan. Sugars and oligosaccharides such as lactose, lactulose, raffinose, stachyose and fructo-oligosaccharides (FOS) also escape absorption in the small intestine and are metabolized by species of colonic bacteria. Mucin glycoproteins, which are produced by goblet cells in the colonic epithelium, are predominant endogenous substances fermented in the colon. Related mucopolysaccharides such as chondroitin sulphate and heparin, and pancreatic and bacterial secretions, are also available for the intestinal microflora. Finally, proteins and peptides originating in the diet, in pancreatic secretions or produced by bacteria are also available, although to a lesser extent than the carbohydrates (Manning and Gibson, 2004).

1.1.3 Factors that influence prebiotics properties of carbohydrates

The prebiotic properties of carbohydrates are likely to be influenced by the following factors:

1. Monosaccharide composition: Recognized prebiotics are built primarily from glucose, galactose, xylose and fructose.

2. Glycosidic linkage. The linkage between the monosaccharide residues is a crucial factor in determining both selectivity of fermentation and digestibility in the small intestine. Fermentation of FOS prebiotics is selective because of a cell-associated β-fructofuranosidase in the bifidobacteria.
3. Molecular weight Polysaccharides are generally not prebiotic in their metabolism but oligosaccharides have this property. Inulin has the highest molecular weight, but most of the carbohydrate in inulin has a degree of polymerization less than 25, with an average of about DP 14. The effect of molecular weight on prebiotic properties can be seen also for that xylans and pectins .

   Most current prebiotics are of relatively small DP, the exception being inulin. It is thought that the oligosaccharides must be hydrolysed by cell-associated bacterial glycosidases prior to uptake of the resultant monosaccharides. It is, therefore, reasonable to assume that to a longer chain of the oligosaccharide could be associated a slower the fermentation and hence the further the prebiotic effect will penetrate more effectively throughout the colon. For example, long-chain inulin may exert a prebiotic effect in more distal colonic regions compared with the lower-molecular-weight FOS, which may be more quickly fermented in the saccharolytic proximal bowel.

1.2 Oligosaccharides

The carbohydrates can be classified according to their molecular size or degree of polymerization (number of monosaccharide units combined), into monosaccharides, oligosaccharides or polysaccharides. According to IUBI-UPAC nomenclature, oligosaccharides are defined as oligosaccharides containing between 3 and 10 sugar moieties (Mussatto and Mancilha, 2007). Consequently, oligosaccharides are low molecular weight carbohydrates. At the same time, based on the physiological properties, the carbohydrates can be classified as digestible or non-digestible (NDOs). The main categories of NDOs presently available or in development as food ingredients include carbohydrates in which the monosaccharide unit is fructose, galactose, glucose and/or xylose.

The NDOs are known to promote the growth of beneficial bacteria in the colon, mainly the *Bifidobacteria* species, and are thus recognized as prebiotics. Sako, et al, 1999, described 13 classes of NDOs that present bifidogenic functions, and are commercially produced (Table 1). The chemical

differences among these NDOs include chain length, monosaccharide composition, degree of branching, and purity.

NDOs of various types can be found as natural components in milk, honey, fruits and vegetables such as onion, Jerusalem artichoke, chicory, leek, garlic, artichoke, banana, rye, barley and salsify. For most of these sources, concentrations range between 0.3% and 6% of fresh weight; for chicory and salsify these values are between 5% and 10% while in Jerusalem artichoke they can reach up to 20%. Other examples of naturally occurring non-digestible oligosaccharides are the galactosylsucroses arabinose and stachyose in soybean and other pulses and leguminous seeds, xylooligosaccharides in bamboo shoots and galactose-containing oligosaccharides in milk, particularly colostrums either in free form or as glycoconjugates (Voragen, 1998).

Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato and rye are special sources of fructooligosaccharides (Sangeetha, et al, 2005; Yun, 1996, Ziemer and Gibson, 1998). Isomaltulose naturally occurs in honey, sugarcane juice, and products derived thereof such as treacle or food-grade molasses (Lina et al, 2002).

Xylooligosaccharides appear naturally in bamboo shoots, fruits, vegetables, milk and honey (Vázquez, 2000) .In Figure 1 it is reported a schematic representation of the principal non-digestible oligosaccharides obtained from foods by process as extraction and hydrolysis.

Table 1. Non-digestible oligosaccharides with bifidogenic functions commercially available (Sako et al, 1999)

Compound	Molecular structure[a]
Cyclodextrins	$(Gu)_n$
Fructooligosaccharides	$(Fr)_n$–Gu
Galactooligosaccharides	$(Ga)_n$–Gu
Gentiooligosaccharides	$(Gu)_n$
Glycosylsucrose	$(Gu)_n$–Fr
Isomaltooligosaccharides	$(Gu)_n$
Isomaltulose (or palatinose)	$(Gu–Fr)_n$
Lactosucrose	Ga–Gu–Fr
Lactulose	Ga–Fr
Maltooligosaccharides	$(Gu)_n$
Raffinose	Ga–Gu–Fr
Soybean oligosaccharides	$(Ga)_n$–Gu–Fr
Xylooligosaccharides	$(Xy)_n$

[a] Ga, galactose; Gu, glucose; Fr, fructose; Xy, xylose.

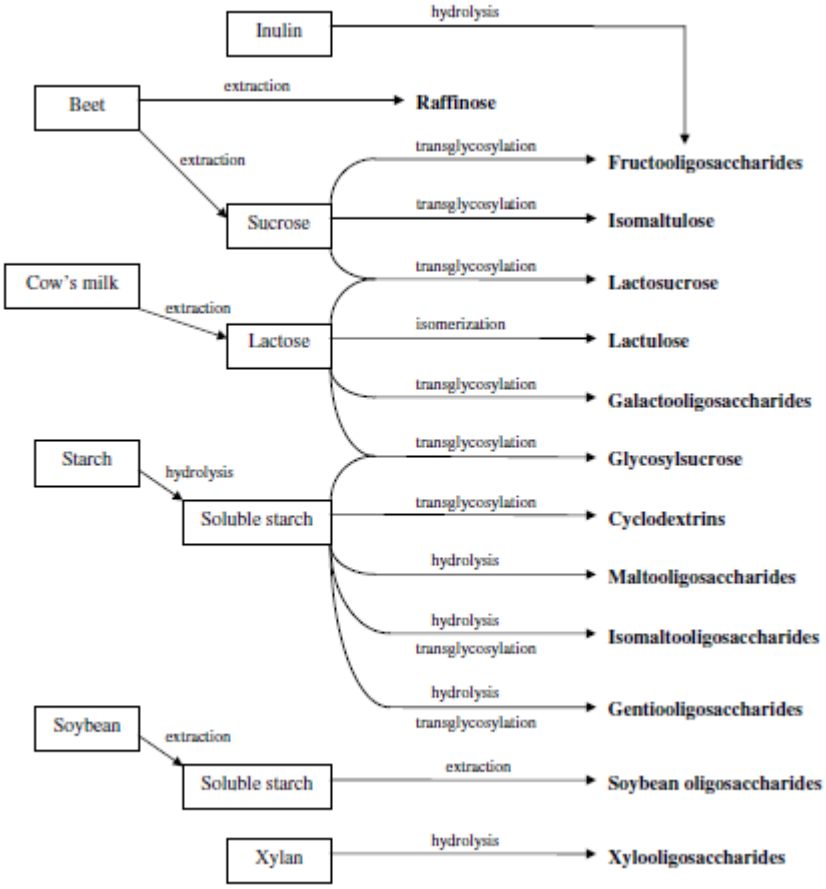

Figure 1-Schematic representation of production processes of non-digestible oligosaccharides (Sako et al, 1999)

1.3 α-Galactosides

α -Galactosides of sucrose, namely raffinose, stachyose and verbascose and ajugose are widely distributed in higher plant, especially leguminous where they accumulate in high concentration in the storage organs and they accumulate in high concentration in the storage organs and they have the function as storage carbohydrates during germination. They are composed of α-(1→6) galactosides bound to sucrose (β-D-fructofuranosyl-(1→2) α-D-glucopyranoside) at C6 of the glucose moiety (Andersen et al, 2003) and they have respectively one (raffinose), two (stachyose), three (verbascose) and four galactosyl units. The galactosylation occurs in through transfer from a myo-inositol derivative, galactinol.

In soybeans and in some legums, and in Brassica campestris there is in addition in the presence of galactopinitol, galactinol, mannitriose and melibiose (Naczk et al, 1997). Due to the absence of galactosidase activity in human and animal intestine mucosa, they pass into the large intestine, where bacterial enzymes decompose them into short fatty acids and gases. The negative effect of flatulence is counterbalanced by an increasing interest in non-digestible oligosaccharides as functional food ingredients. They have several health benefits, such as lowering blood cholesterol, reducing blood pressure and preventing some types of cancer (Roberfroid, M, 2007; Tomomatsu, H 1994). In literature there are also cited for their prebiotics properties (Dinoto et al, 2006; Matteuzzi et al, 2004).

Analytical methods employed for the determinations of α-Galactosides in leguminosae are enzymatic method (Maughan et al, 2000),. high-peformance capillary electrophoresis (Andersen et al, 2003), high performance size exclusion chromatography with refractive index detection (HPSEC-RI) and HPAEC-PAD (Bainy et al, 2008).

E.Giannocaro et al, 2008 compared HPLC and enzymatic methods: the enzymatic method has the advantage that it doesn't require expensive instrumentation but it doesn't permit the quantification of individual sugars such as raffinose and stachyose. Between the two HPLC methods HPAEC-PAD is more sensitive, faster and with an higher peak resolution than HPSEC-RI.

1.4 Isomaltooligosaccharides

Isomalto-oligosaccharides (IMO) are composed of glucose monomers linked by α (1–6)- glucosidic linkages (Gibson, 2004) Isomaltooligosaccharides (IMO) consist mainly of isomaltose, isomaltotriose, panose, isomaltotetraose, isomaltopentaose, nigerose, kojibiose, isopanose and other higher branched oligosaccharides. Isomalto-oligosaccharides are produced using starch as the raw material, as reported in Figure 2.IMO act to stimulate the growth of *Bifidobacterium* and *Lactobacillus* species in the large intestine:Numerous research efforts have confirmed the significant efficacy of isomaltooligosaccharides as prebiotics (Chung and Day, 2004; Thitaram et al, 2005, Zhang et al, 2003). The IMO fermentation maintained a lactic acid flora whilst also allowing the generation of butyrate.(Olano-Martin et al, 2000).

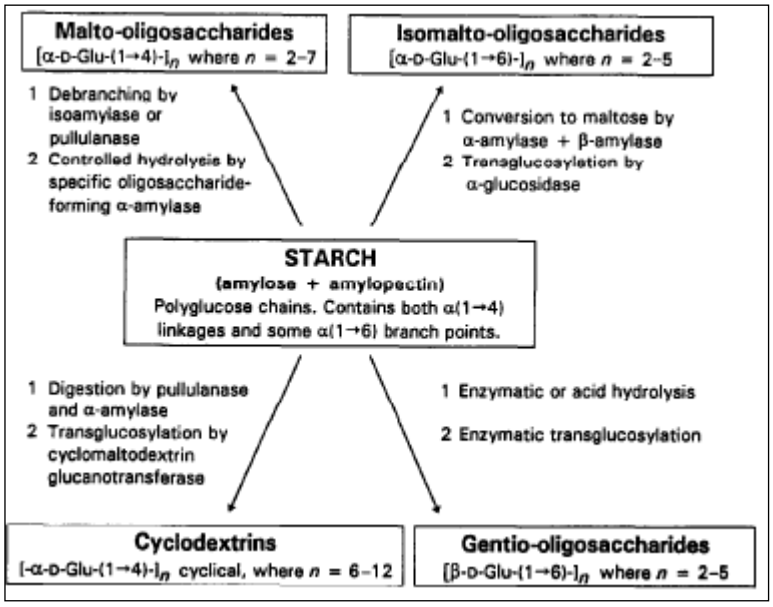

Figure 2. Oligosaccharides manufactured from starch (Glu = glucose). Isomalto-oligosaccharides also contain some a(1 +4) glycosidic linkages (Crittenden and Playne, 1996)

1.5 .Fructans

Fructans is a general term used for any carbohydrate consisting of β-(2→1) fructosyl-fructose linkages. Fructans are characterized by the degree of polymerization (DP) either as the average (DP_{av}) or the maximum (DP_{max}) value. The term inulin-type fructans includes native inulin (DP 2-60, DP_{av}=12), inulin HP (DP 10-60, DP_{av}25) that is a inulin without oligomers with DP<10 eliminated by physical separation techniques or as result of partial enzymatic hydrolysis (DP 10-60, DP_{av}=25), oligofructose (DP 2-7, DP_{av}=4) and a specific combination of oligofructose and inulin HP, named Synergy.

Oligosaccharides with DP lower than are sub classified in two series: inulooligosaccharides (IOS) and fructooligosaccharides (FOS). The first series is only composed by fructose unit chains also called FpyFn (*β*-D-fructofuranosyl-[*α*-D-fructofuranodyl] $_{n-1}$-D-fructofuranoside) while the second series has also a terminal glucose unit and can be called GpyFn (*α* -D-glucopyranosyl-[*β* -D-fructofuranosyl] n-1-D fructofuranoside) (Roberfroid, 2007).

Therefore, the general formula may be depicted as Fn (for inulooligosaccharides) or GFn (for fructooligosaccharides), with G as glucose and F as fructose, and n characterizing the total number of

units (Ronkart et al, 2007). Composition depends on the plant source, harvesting date but also on extraction and post-extraction processes (Praznik and Beck, 1985; De Leenheer, 1994). Chicory fructooligosaccharides are present in significant amounts in several edible fruits and vegetables. Average daily consumption has been estimated to be between 1 and 4 g in the United States and between 3 and 11 g in Europe.

1.6 Fiber gums

Fiber Gums are often used in such foods as yogurt to cause the product to have a thicker consistency. They can be used as a prebiotic food or as construction material. Obviously, processing varies according to the desired outcome.

Fiber gums are water-soluble and derived from such plants as acacia, carrageenan, guar, locust bean, and xanthan. Usually containing about 85% fiber, these gums help promote the production of large quantities of short-chain fatty acids, which are known to play several beneficial roles, including the development of such intestinal bacteria as *Lactobacillus* and *Bifidobacteria*.

Studies have shown that fiber gums do not cause the diarrhea and flatulence often associated with FOS intake, even at high doses.

1.7 Lactitol

Lactilol is a disaccharide alcohol analogue of lactulose. Lactilol is used in many countries for treating constipation and hepatic encephalopathy, but not so in the United States. In Japan, lactilol is also used as a prebiotic because it is resistant to digestion in the upper gastrointestinal tract and is fermented by a limited number of colonic bacteria. However, it is not approved as a prebiotic in the United States either. In Europe, it is used as a food sweetener.

1.8 Lactosucrose

Lactosucrose is a trisaccharide comprised of galactose, glucose, and fructose molecules. It is produced through enzyme action that results in sucrose. Resistant to digestion in the stomach and small intestine,

lactosucrose acts on the intestinal microflora to increase significantly the growth of the *Bifidobacterium* species.

Lactosucrose is widely used in Japan as a dietary supplement and in functional foods, including yogurt and is being developed in the United States for similar uses.

1.9 Lactulose

Lactulose is a semi synthetic disaccharide comprised of lactose and fructose. Lactulose is resistant to human digestive enzymes and is fermented by a limited number of bacteria in the colon, especially *Lactobacilli* and *Bifidobacterium*. Currently, lactulose is a prescribed drug in the United States for the treatment of constipation and hepatic encephalopathy, but it is still in experimentation to see if it is really a prebiotic substance. In Japan, it is marketed as a dietary supplement and for use in functional foods. Lactulose has exhibited some ability to reduce infectious inflammatory bowel disorders, as well as some colonic tumors. Since it has some ability to improve glucose tolerance and is showing other improvements on carbohydrate metabolism, it is speculated that Lactulose may be helpful in treating diabetes mellitus. In addition, it has significantly stimulated calcium absorption in postmenopausal women in preliminary clinical work. One cautionary note is that some lactulose preparations contain galactose and would be contraindicated in those who require a low galactose diet. In addition, those who are lactose intolerant should avoid the use of lactulose, lactilol and transgalacto-oligosaccharides.

1.10 Oligofructose

Oligofructose is a sweet product derived from native inulin and is about 30-60% as sweet as sugar. It is found on the market as an oligosaccharide because it consists mainly of fructose units with some glucose-terminated chains. It is also available as a mixture with inulin to reduce the amount of non-glucose terminated chains. The unbound fructose chains have prebiotic properties but with a different fermentation profile than either inulin or FOS. However it is fermented by a wider variety of probiotic bacteria than inulin. Unlike inulin, oligofructose has the ability to brown, making it a valuable addition to baked products.

1.11 Transgalacto-oligosaccharides

TOS (transgalacto-oligosaccharides) are a mixture of glucose and galactose oligosaccharides. They are produced from lactose via enzyme action obtained from *Aspergillus oryzae*, which can also be a pathogen. TOS are resistant to digestion in the upper gastrointestinal tract, thereby able to stimulate the growth of bifidobacteria in the large intestine. TOS are marketed in Japan and Europe as dietary supplements and used in functional foods. They are being developed for similar use in the United States. TOS have demonstrated positive effects on calcium absorption and have prevented bone loss in some animal research. In preliminary studies, TOS have shown some ability to lower triglycerides.

1.12 Xylo-oligosaccharides

Xylo-oligosaccharides are comprised of oligosaccharides containing beta-linked xylose residues. Obtained from enzymatic action, they are marketed in Japan as prebiotics and are being developed for similar use in the United States. Since xylo-oligosaccharides resist digestion in the upper gastrointestinal tract, they are able to function in the large intestine to increase the growth of *Bifidobacterium* species, thus improving gastric function. According to preliminary research, xylo-oligosaccharides have the potential to improve blood sugar levels and fat metabolism, restore normal intestinal flora following antibiotic, chemo, or radiation therapies, increase mineral absorption and vitamin B production, and reduce intestinal purification.

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2. HIGH PERFORMANCE ANION EXCHANGE CHROMATOGRAPHY WITH PULSED AMPEROMETRY DETECTION (HPAEC-PAD)

2.1 HPAEC-PAD advantages and comparison with other techniques for carbohydrates analyses

Conventional techniques for sugars determination are founded on separations on either amino-bonded silica or metal-loaded cation exchange columns coupled with refractive index detection (RI) applied to dietary fiber determinations. Although useful for some purposes, these techniques are not useful for separating the mixtures of all carbohydrates present in nonstarch polysaccharides (NPS).

They are also limited by the non specific nature and relative low sensitivity of RI compared with the flame ionization detector (FID) in GLC (Sungsoo Cho and Dreher, 2001).

The advent of high-performance anion-exchange chromatography system (HPAEC) in the late 1980s revolutionized carbohydrates analyses. With the intent to provide a highly sensitive detection and efficient separation tool for carbohydrates, a very happy marriage between HPAEC and PAD (pulsed amperometric detection) was exploited. This technique is successfully employed for carbohydrates analyses; the major advantages are that it doesn't require any derivatization process with minimal sample preparation and cleanup and it is a very selective and sensitivity technique. Furthermore HPAEC-PAD compared to other HPLC or GC methods, is compatible with gradient elution and permits to separate multicomponent mixture containing monosaccharides, disaccharides, oligosaccharides (Cataldi et al, 2000), providing to be a reliable tool in fundamental research on dietary fiber characterization.

The principal "strength" of HPAEC-PAD technique is due to the possibility to separate anomeric and positional isomers, including for example, the distinction between F series from GF series.(F=fructose, G=glucose) (Borromei et al, 2009) .

HPAEC-PAD permits the separation of sugars, sugar alcohols and oligo-and polysaccharides with high resolution in a single run and quantities at the picomole level if necessary. HPAEC-PAD has been

successfully applied to food, dietary fiber, and complex carbohydrate analysis and it also widely used for glycoprotein research (Lee , 1996, Cataldi, et al, 2000).

2.2 Meccanism of separation and Carbopac column choice

HPAEC chromatography takes advantage of the weakly acidic nature of carbohydrates to give highly separations at high pH using a strong anion-exchange stationary phase.

At high pH carbohydrates at least partially ionized and thus can be separated by anion-exchange mechanism. This approach cannot be used with classical silica-based columns because these matrices dissolve at high pH.

The polymer-based matrices used in anion-exchange chromatography are characterized by high mechanical and chemical stability. Depending on the category of compounds, suitable columns can be chosen differing in capacity, resin composition, cross-linking and organic solvent compatibility.

For example for mono and disaccharides analysis, the Carbopac PA 1 Dionex is employed, this column is packed with a polystyrene/divinylbenzene substrate agglomerated with a Microbead ™ quaternary amine funzionalized latex (Table 1).

Another column suitable for mono and disaccharides analysis is Carbopac PA10, which is similar to Carbopac PA1 but holds a higher percentage of divinylbenzene.

For oligosaccharides resolution and separations another pellicular anion- exchange column is successfully optimized, designed as Carbopac PA100 that it is packed with a macroporous resin obtained from the copolymerization of ethylvinyl and divinylbenzene and it possesses a much higher compatibility with organic solvents. A more recent column suitable for oligo and polysaccharides analyses is Carbopac PA200, the resin consists of 5.5-µm diameter non-porous beads covered with a fine latex of functionalized MicroBeads. This pellicular resin structure permits excellent mass transfer, resulting in high-resolution chromatography. Ideal for fast, high resolution profiling of homologous sugar series such as inulins, amylopectins, and malto- oligosaccharides.

Finally a stationary phase especially designed for the separation of alditols is the column Carbopac MA1 that it is packed with a macroporous polymeric resin which has an ion exchange capacity 45 times greater than that of the Carbopac PA1.

Mechanisms of carbohydrate separation is closely related to pk_a of the analytes; therefore it is possible to separate an acidic carbohydrate respect than the respective neutral (for example mannose-6-phosphate respect mannosFor homologous carbohydrates, the retention time is related to the number of carbons (or hydroxyl groups attached to them). In a homologous oligosaccharides series the order of elution depends on the degree of polymerization (DP).

Table 1. Comparison of the Carbopac MA1, PA1 and PA100 (Dionex).

Characteristic	CarboPac MA1	CarboPac PA1	CarboPac PA100
Recommended applications	Mono- and disaccharide alcohol analysis in food products, physiological fluids, tissues, and reduced glycoconjugate saccharides[b]	Monosaccharide compositional analysis, linear homopolymer separations, saccharide purification	Oligosaccharide mapping and analysis
Resin composition	8.5-µm-diameter vinylbenzyl-chloride/divinylbenzene macroporous substrate fully functionalized with an alkyl quaternary ammonium group	10-µm-diameter polystyrene/ divinylbenzene substrate agglomerated with 350-nm MicroBead quaternary amine functionalized latex	10-µm-diameter ethylvinylben-zene/divinylbenzene substrate agglomerated with 350-nm MicroBead quaternary amine functionalized latex
MicroBead latex cross-linking	N/A, no latex	5% cross-linked	6% cross-linked
Anion-exchange capacity	4500 µeq per 4 × 250-mm column	100 µeq per 4 × 250-mm column	90 µeq per 4 × 250-mm column
Recommended flow rate	0.4 mL/min (4 × 250-mm column)	1 mL/min (4 × 250-mm column)	1 mL/min (4 × 250-mm column)
pH compatibility	pH 0–14	pH 0–14	pH 0–14
Organic solvent compatibility	0%	0–2%	0–100%
Maximum back pressure	2000 psi (14 MPa)	4000 psi (28 MPa)	4000 psi (28 MPa)

[b] Note that sialylated and other acidic mono- and oligosaccharides may not be recovered from the CarboPac MA1 column. It is not recommended that this column be used with these analytes.

Positional isomers (for example isomaltose and maltose) and anomeric isomers (for example maltose and cellobiose) are also well separated (Lee, 1996).

2.3 Eluent composition

The mobiles phase composition in HPAEC-PAD significantly influences the selectivity and rapidity of separation as well as the sensitivity of detection. Sodium hydroxide solutions are normally employed with a variable OH^- concentration, depending on the class of the compounds under investigations. In

the preparation of sodium hydroxide solution it is extremely important to minimize contamination of the eluent solutions with carbonate. Carbonate, being a divalent anion at pH 12, binds strongly to the columns and interferes with carbohydrate binding, causing a drastic decrease in column selectivity and a loss of resolution and efficiency.

Sodium acetate or sodium nitrate are generally added to the mobile phase as "pushers"; these anions are able to interact more strongly than hydroxide with the anion-exchanges sites and the retention time can be drastically decreases. These ions are often employed in gradient elution where the separation of complex mixture of oligosaccharides with different sizes and acidities has been carried out. Wong and Jane , 1994, reported that the use of nitrate as pushing agent gives a higher resolution and a greater sensitivity than acetate. Furthermore nitrate have an higher affinity for strong anion-exchange resins, therefore a lower NaOH concentration may be used for elution, which reduces the chances of epimerization and degradation when alkali-sensitive terminal sugars are present in the oligosaccharide.

2.4 Pulsed amperometry detection:theory of operation

Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode. The products of this oxidation reaction also poison the surface of the electrode, which means that it has to be cleaned between measurements.

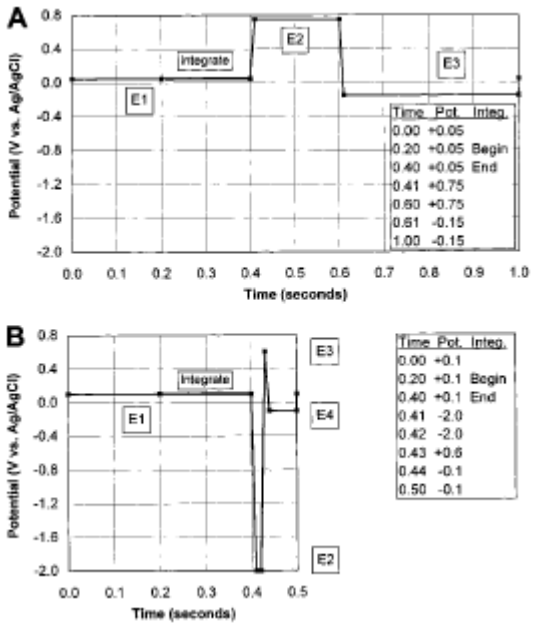
This is accomplished by first raising the potential to a level sufficient to oxidize the gold surface. This causes desorption of the carbohydrate oxidation products. The electrode potential is then lowered to reduce the electrode surface back to gold.

Firstly, a repeating sequence of three potentials are applied for pulsed amperometric detection. In this application, current from carbohydrate oxidation is measured at the first potential, E1. The second, E2, is a more positive potential that oxidizes the gold electrode and cleans it of products from the carbohydrate oxidation. The third potential, E3, reduces the gold oxide on the electrode surface back to gold, thus permitting detection during the next cycle at E1. The three potentials are applied for fixed durations referred to as t1, t2, and t3. The step from one potential to the next produces a charging current that is not part of the analyte oxidation current, so the analyte oxidation current is measured after a delay that allows the charging current to decay. The carbohydrate oxidation current is measured

by integrating the cell current after the delay (Technical Note 20 Dionex). In 1998, Rocklin et al., introduced a new quadruple-potential waveform for detection of carbohydrates using pulsed amperometry (Figure 1). The new waveform cleans the electrode by application of a potential more negative than the potential limit. In contrast to a commonly used triple-potential waveform, negative cleaning allows the time during which gold oxide is formed to be minimized, thus minimizing the dissolution and resulting recession of the gold working electrode as a result of gold oxide formation/reduction cycles.

Preventing gold electrode recession is shown to improve long-term reproducibility. Compared to the triple-potential waveform, the quadruple-potential waveform shows similar minimum detection limits but greatly improved long-term reproducibility.

Figure 1. Standard (A) and quadruple-potential (B) waveform for pulsed amperometric detection of carbohydrates. Detector response in the charge (in coulombs) from integration of the carbohydrate oxidation current between 0.2 and 0.4 s.



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3. STUDY OF *Saccharomyces cerevisiae sp.* METABOLISM ON A SUBSTRATE OBTAINED FROM DEFATTED *Leguminosae* SEEDS.

3.1 Introduction

3.1.1 Regulations about sugar industry

Regulation (EC) No 318/2006 on the common organization of the markets in the sugar sector and Regulation (EC) No 320/2006 established a temporary scheme for the restructuring of the sugar industry in the Community. In fact, due to developments within the Community and internationally, it was necessary to adjust the production system in order to provide for new arrangements and reductions of the quotas. Consequently, in Italy 16 sugars refinery of 21 are closed because they are not competitive with international production.

This leads for industry of yeasts to the need to research a new sugary substrate in substitution of traditional molasses.

The yeast *Saccharomyces cerevisiae* is commonly known as "baker yeast" or brewer's yeast". The yeast ferments sugars present in the flour or added to the dough, giving off carbon dioxide (CO_2) and alcohol (ethanol). The CO_2 is trapped as tiny bubbles in the dough, which rises.

3.1.2 Metabolism of Saccharomyces cerevisiae

Saccharomyces cerevisiae is a facultative aerobic fermenter . The major source for energy production in the yeast, *Saccharomyces cerevisiae*, is glucose and glycolysis is the general pathway for conversion of glucose to pyruvate, whereby production of energy in form of ATP is coupled to the generation of intermediates and reducing power in form of NADH for biosynthetic pathways (Figure 1).

It also was found that *S. cerevisiae (*Seung-Heon, et al, 2003) partially removed some di- and trisaccharides, but did not remove certain, less common monosaccharides and some common disaccharides, like lactose. In Table 1 it is reported which carbohydrates are fermented by

Saccharomyces cerevisiae cells and by which type of enzymes. .(Fraenkel, 1982, Fraser et al, 1997, Kim and Robyt, 199,Robyt, 2000, Truscheit, 1981, Yoon and Robyt, 2002)

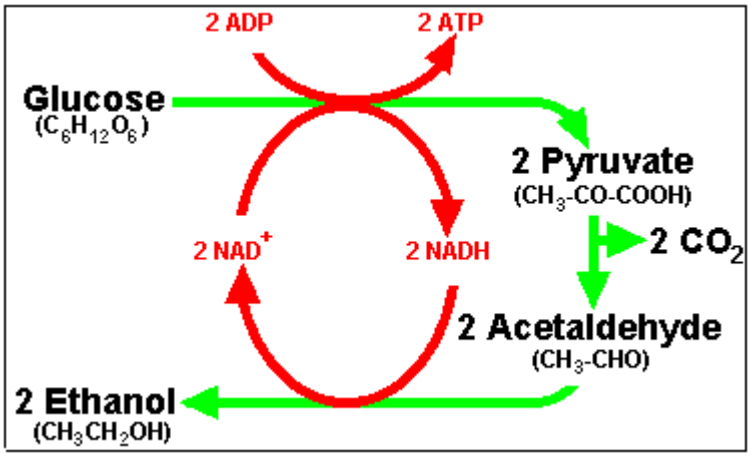

Figure 1. Glycolysis

Table 1. Type of carbohydrates that are fermented by *Saccharomyces cerevisiae* sp

Carbohydrates	Substrate	Fermentation	Enzymes	Products
Monosaccha-rides	Glucose	Yes		
	Fructose	Yes		
	Mannose	Yes		
	Galactose	Yes		
	Melibiose	No		
	Xylose	No		
	Ramnose	No		
	Fucose	No		
	L-sorbose	No		
	Quinivose	No		
Disaccharides	Maltose	Yes	Maltase	glucose+fructose
	Sucrose	Yes	Invertase	glucose+fructose
	Turanose	Yes	Glucosidase	glucose+fructose
	Melibiose	Yes	Melibiase	glucose+galactose
	Isomaltose	Yes (slowly)	Glucosidase	glucose+glucose
	Trehalose	Partially	Trehalases	glucose+glucose
	Cellobiose	No		
	Lactose	No		
Trisaccharides	Raffinose	Yes (slowly)	Invertase+melibiase	Glucose+galactose+fructose

	Maltotriose Isomaltotriose	Partially No	Glucosidase	glucose+glucose+glucose
Tetrasaccha-rides	Maltotriose Isomaltotriose	No No		
Oligosaccha-rides	Maltodextrins and isomaltodextrins	Only with molecules of glucose < 3-4		

3.1.2 Leguminosae seeds composition

Legume foods are a rich source of protein and amino acids (like glutammic acid, aspartic acid and arginine), carbohydrate, vitamins (like thiamin, niacin, riboflavin, vit. C, vit. A (β-carotene), tocopherols and minerala (like calcium, iron, zinc, copper and selenium), with good storage properties. Composition of leguminosae seeds depends varies among sub-families and varieties.

For example the total amount of soluble sugars in soybean seeds varying from 6.2% and 16.6% , with a major quantity of sucrose, from 3 to 10% that is responsible for enhancing the sweet taste of soybean (Taira, 1975), followed by stachyose (0.6-5.8%) and raffinose (0.1-1.8%) (Trugo et al, 1995). Dietary fiber is in the range from 13.7÷16.5 g/100g for yellow soybean; 9.19÷9.45 g/100g for green soybean.

Raffinose and stachyose are α-galactosides, which the chemical structure and prebiotic functions are reported in Chapter 1.

The presence of α-galactosides in leguminous, as well as others antinutrients (like trypsin inhibitor, phytic acid, saponins, phytoheamagglutins and tannins) decreases the bioavailability of trace elements and proteins (Shimelis and Rakshit, 2007).

The raffinose family oligosaccharides are reserve carbohydrates that are stored in the leguminous endosperm, in particular in the vacuoles, while galactomannans are stored in the endospermic cell walls (Buckeridge, Dietrich, 1996). α-galactosides are accumulated in legume seeds during maturation and are then used as an energy source during germination; furthermore they are also related to the ability of legume seeds to survive when exposed to desiccation (Ekvall et al, 2006). Galactomannans are considered “multipurpose macromolecule” because they are considered an important factor for the adaptation of seeds to different climates (Buckeridge et al, 1995).

3.1.3 State-of-art

α-galactosides are found and studied in many leguminosae seeds, like lupins (Lupinus family) (Muzquiz et al, 1999; Andersen et al, 2003), soy (*Glycine max* (L.)Merr) (Bainy et al, 2008; Obendorf et al, 2008; Giannoccaro et al, 2008, peanuts (*Arachis hypogaea L*.) (Rose and Mixon, 1989) beans (*Vicia faba L*.) (Dini et al, 1989, Dey et al. 1982), chipkea (*Cicer arietinum L*.) (Xiaoli et al, 2008*)* peas (*Pisum sativum)* (Ekvall et al, 2006).

Raffinose is also present in molasses that are normally employed in bakery industry as substrate for *Saccharomyces cerevisiae* growth. Raffinose synthesis is catalyzed by raffinose family synthase, a trasferase that catalyzes the condensation of myo-inositol with UDP-galactose. The synthesis of galactinol is catalyzed by galactinol synthase, a transferase that catalyzes the condensation of myo-inositol with UDP-galactose. Raffinose accumulation in sugarbeet depends from many factors, like storage duration, harvest date, and storage temperature (Hageenson, et al, 2008).

Determination of α-galactosides of the raffinose family, like for the other carbohydrates studied in this thesis, is complicated by the fact that they neither absorb ultraviolet or visible light nor fluorescence. Derivatization of carbohydrates with a suitable chromophore or fluorophore is thus an often-used technique to improve the detection limit. However, most derivatization schemes are based on the reducing properties of sugars, a property that α-galactosides do not posses. In literature are reported enzymatic, GC , HPLC-RI, HPAEC-PAD (Giannoccaro et al, 2008; Knudsen and Li, 1991, Bainy et al, 2008),and capillary electrophoresis methods (Andersen et al, 2003) To quantify α-galactoside . HPLC has become the preferred method because it permits a simple and efficient separation of these carbohydrates, without derivatization. Between RI detection commonly used in leguminosae sugars analyses (Kim et al, 2008, Johaensen et al, 1996) and PAD, this is preferred because is highly selective and more sensitive than RI (Giannoccaro, E, 2008).

3.1.4 Aim of this work

This work was focused on the research of an alternative sugary substrate in substitution of molasses for *Saccharomyces cerevisiae sp*. growth.

In a first moment optimization of the industrial extraction procedure was performed in terms of the choice of pH, solvent/substrate ratio and number of extraction steps needed to obtain the maximum carbohydrate content.

With the aim to identify carbohydrates and oligosaccharides distribution, preliminary analyses were conducted by a Ion Trap Mass spectrometry.

Due to the limit of Mass spectrometry that it doesn't permit to distinguish oligosaccharides with the same degree of polymerization, optimization of an HPAEC-PAD method was necessary.

This method was validated following Eurachem guide, 1998**,** with the purpose to perform qualitative and quantitative analyses on carbohydrates identified.

Finally, a study on the metabolism of yeast was carried out in order to verify that the carbohydrates present in the extract could be employed by *Saccharomyces cerevisiae* cells.

3.2 Materials and methods

3.2.1 Sample preparation

Standard stock solutions of glucose, fructose, sucrose, raffinose, stachyose, verbascose (Sigma) were prepared at a concentration of 5000 ppm.

Leguminosae panels were defatted, then carbohydrates were extracted with water in a ratio 1:10 solvent/substrate at the temperature of 70°C.

The stage of extraction was repeated until 8 times to verify if the process was exhaustive. Then panels with different pH were tested.

Study on metabolism was performed incubating *Saccharomyces cerevisiae* cells on leguminosae panels at 30°C under continuous agitation. 5 mL of samples were collected after 10, 20, 40, 60, 90, 120, 180 and 360 minutes from incubation start.

All samples were dilued with water for HPLC in a ratio 1:3 , and pH was corrected with sodium hydroxide until pH=11, in order to block enzymatic reactions.Solutions were purified by anion exchange SPE columns and filtered by 0.22μm nylon filters first injection into HPLC.

3.2.2 HPAEC-PAD analyses

All carbohydrate analyses, discussed in this thesis, were performed with a Dionex consisted of a GP50 low-pressure quaternary gradient pump equipped with a pulsed electrochemical detector (ED 50) consisting of an amperometric flow through cell and a silver-silver chloride reference electrode.

The ED 50 detector delivered to the electrochemical cell the following potential waveform: E1= 0,1 V (t1=0.20-0.40s), integration from 0.20 and 0.40s, E2=-2.0 V (t2= 0.41-0.42s); E3= 0.6V (t3=0.43s); E4=-0.1 V (t4=0.44-0.50s).

Mobile phase employed are deionized water for HPLC, sodium hydroxide (50% v/v) and sodium acetate.

All mobile phases were sparged and pressurized with helium to prevent adsorption of atmospheric carbon dioxide and subsequent production of carbonate, which would act as displacing ion and shorten retention time.

3.2.3 Mass spectrometry analyses

Mass-spectrometry analysis were conducted by a Ion Trap Mass Spectrometer with ESI source (Agilent 633 Ion Trap), with these parameters:

- direct infusion
- negative mode
- full scan (150÷1500)
- pressure nebulizer:15.0 psi
- dry gas:8 L/min
- gas temperature: 200°C
- Max accuration time: 150000µs
- ICC target: 100000.

Samples were solubilized in H_2O:ACN mixture (50:50 v/v)+ 0.1% HCOOH to favor the ionization in negative mode.

3.3 Result and discussion

3.3.1 Optimization of HPAEC-PAD analyses and preliminary MASS SPECTROMETRY analyses

Preliminary mass spectrometry analysis had permitted to identify degree of polymerization of carbohydrates extracted from leguminosae panel seeds (Figure 2). Carbohydrate signals are obtained as $[M]^-$ and as $[M+HCOO]^-$: disaccharides, trisaccharides and tetrasaccharides signals are circled in Figure 2. Pentasacccharides are probably present but the correspondent signal to noise is very low.

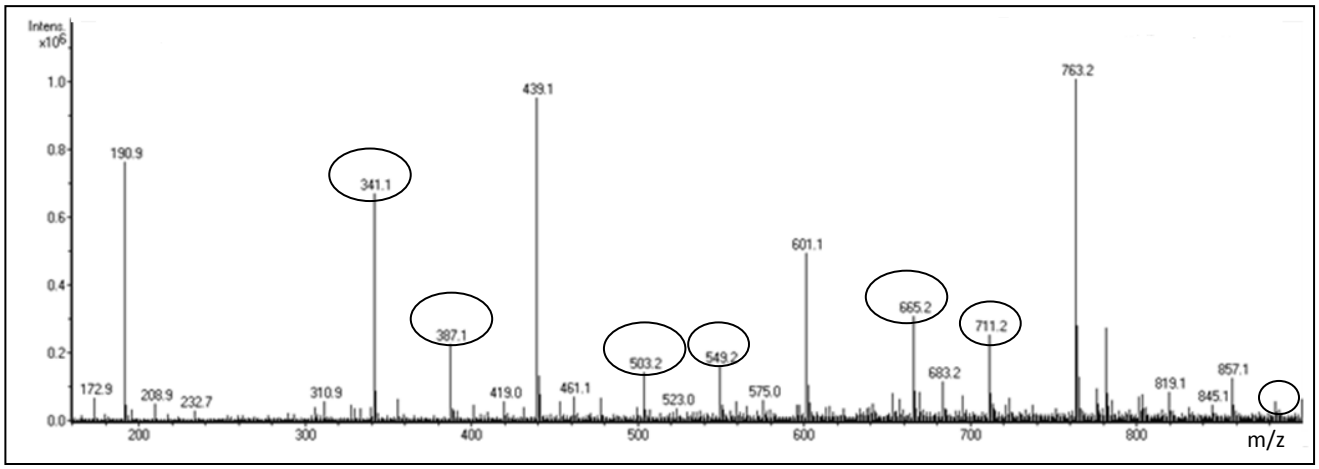

Figure 2-Mass spectrometer of a leguminosae panel

From this analysis no notices about isomers are possible to obtain, therefore HPAEC-PAD analyses were conducted with the aim to identify them.Two columns were tested for carbohydrates analyses: the first one , Carbopac PA20 (4X250mm) with the pre-column, more specific for oligosaccharides with lower degree of polymerization and Carbopac PA100 (3X150mm) with the pre-column, more specific for oligosaccharides and polysaccharides with an higher degree of polymerization. For analyses performed by column Carbopac PA20, the following method was optimized, described in Table 2 and the resulting chromatogram was reported in Figure 3.

Table 2. Column: Carbopac PA20. Flow: 0.5 mL/min

Time (min)	A%(H_20)	B% (NaOH 0.6M)	C% (NaAc 0.5M)
-40 [a]	94	6	0
0	94	6	0
10	94	16	5
25	79	16	7.2
30	50	25	25

[a] Negative time indicates time prior the analysis necessary for column conditioning

Carbopac PA100 resulted better for α-galactosides in terms of peak resolution. The method of elution optimized is reported in Table 3: the first 10 minutes of isocratic conditions are followed by a linear gradient step wher sodium hydroxide was increased from 42mM to 96 mM and sodium acetate from 0 to 75 in 30 minutes. This method had permitted to separate progressively glucose, fructose, sucrose, the trisaccharide raffinose, the tetrasaccharide stachyose and the pentasaccharide verbascose in 24 minutes. The identity of these carbohydrates has been determined by comparison of retention times and spiking with commercial standard (Figure 4).

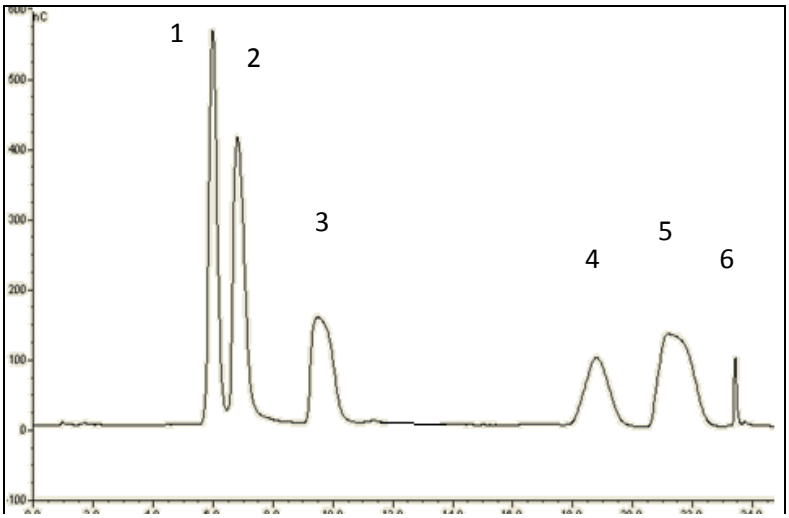

Figure 3. Chromatogram of a standards mixture: 1-glucose; 2-Fructose, 3-Sucrose; 4-Raffinose; 5-Stachyose; 6-Verbascose. a-Standard mixture chromatographic profile.

Table 3. Column: Carbopac PA100 Flow:1mL/min

Time (min)	A%(H_20)	B% (NaOH 0.6M)	C% (NaAc 0.5M)
-40.0 [a]	93	7	0
0.0	93	7	0
10.0	93	7	0
40.0	69	16	15

[a] Negative time indicates time prior the analysis necessary for column conditioning

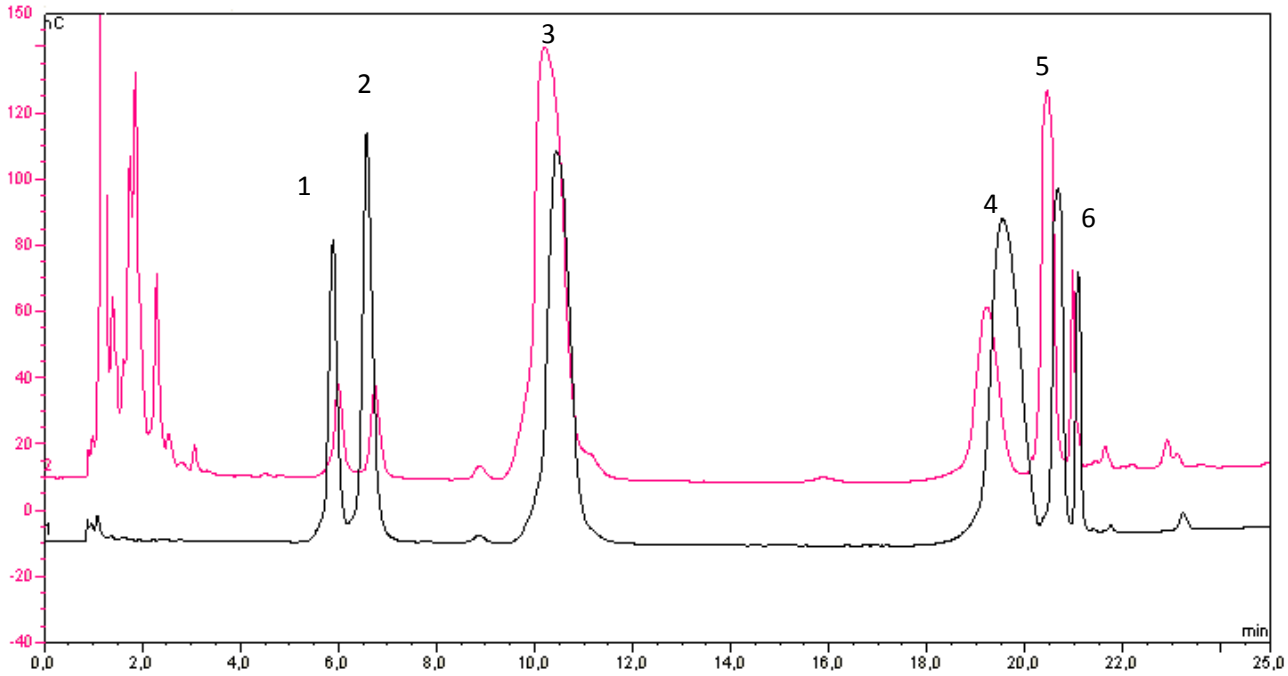

Figure 4. Chromatogram of a standards mixture: 1-glucose; 2-Fructose, 3-Sucrose; 4-Raffinose; 5-Stachyose; 6-Verbascose. a-Standard mixture chromatographic profile; b-Leguminous panel chromatohraphic profile

3.3.2 Validation of HPAEC-PAD method

The optimized method was validated in terms of intraday and interday precision, limit of detection and quantitation, linearity and recovery on blank because a matrix without carbohydrates was not available. Intraday precision was calculated in the same day both in terms of area and retention times performing 10 indipendent replicates of a glucose, fructose, sucrose, raffinose, stachyose and verbascose mixture corresponding to the second level of calibration curve. Coefficient of variation resulted lower than 5.8% regarding area values and 1.3% regarding retention time values (Table 4).

Table 4-Interday precision

Area (nC*min) (n=10)						
	Glucose	**Fructose**	**Sucrose**	**Raffinose**	**Stachyose**	**Verbascose**
Mean value	47.28	41.94	38.20	16.50	20.05	12.08
Std deviation	2.71	2.27	1.80	0.37	0.56	0.55
cv%	**5.73**	**5.41**	**4.71**	**2.25**	**2.79**	**4.55**

Retention Times(min) (n=10)						
	Glucose	**Fructose**	**Sucrose**	**Raffinose**	**Stachyose**	**Verbascose**
Mean value	6.68	8.40	12.28	22.35	23.29	24.16
Std deviation	0.08	0.13	0.14	0.26	0.11	0.10
cv%	**1.24**	**1.52**	**1.11**	**1.15**	**0.49**	**0.40**

Repeatibility was calculated in two different days performing 10 indipendent replicates of a glucose, fructose, sucrose, raffinose, stachyose and verbascose mixture corresponding to the second level of calibration curve.Results about repeatibility, calculated both on area and retention times, are reported in Table 5.

Table 5-Intraday precision

Area (nC*min) (n=20)						
	Glucose	**Fructose**	**Sucrose**	**Raffinose**	**Stachyose**	**Verbascose**
Mean value	80.11	94.57	63.66	29.43	33.55	20.46
Std deviation	3.44	8.24	2.26	1.17	1.73	0.49
cv%	**4.29**	**8.72**	**3.56**	**3.99**	**5.17**	**2.42**

Retention Times (min) (n=20)						
	Glucose	**Fructose**	**Sucrose**	**Raffinose**	**Stachyose**	**Verbascose**
Mean value	6.41	8.00	11.59	21.13	22.87	23.79
Std deviation	0.04	0.05	0.08	0.15	0.05	0.04
cv%	**0.58**	**0.70**	**0.69**	**0.69**	**0.24**	**0.18**

Limit of detection was calculated following this formula:

$$LOD = \frac{3\sigma_b}{b}$$

Where :σ_B = Standard deviation blank signal

b_1 = Slope of calibration curve

Limit of quantitation (LOQ) was calculated following this formula:

$$LOQ = \frac{10 \cdot \sigma_b}{b_1}$$

Limit of detection and quantitation values are reported in Table 6.

Table 6-Limit of detection and quantitation values.

µg/mL	Glucose	Fructose	Sucrose	Raffinose	Stachyose	Verbascose
LOD	**0.04**	**0.01**	**0.09**	**0.04**	**0.08**	**0.17**
LOQ	**0.14**	**0.08**	**0.31**	**0.15**	**0.15**	**0.58**

Linearity was established performing a calibration curve for each standards at 6 six levels of concentration on triplicate. Good linearity was showed with R^2 values equal or higher than 0.990 (Table 7).

Table 7.Curves of calibration

Carbohydrates	Slopes	R^2
Glucose (2÷200 µg/mL)	1.061	0.990
Fructose (2÷200 µg/mL)	0.527	0.991
Sucrose(2÷200 µg/mL)	0.262	0.991
Raffinose(2÷200 µg/mL)	0.823	0.992
Stachyose(1÷241 µg/mL)	0.504	0.990
Verbascose (1÷97 µg/mL)	0.668	0.993

Recovery was calculated by adding three different concentration of each standards to a sample of leguminosae corresponding to 10, 20 and 30% of the amount of the sample.

Recovery was calculated following this formula:

$$\text{Recovery (\%)} = (C1-C2)/C3 \times 100$$

where C1 = concentration determined in fortified sample

C2 = concentration determined in unfortified sample

C3 = concentration of fortification

Percentages of recovery values are resulted in the range from 90.14 and 103.05%, as showed in Table 8.

Table 8. Percentages of recovery values

Recovery	10%	20%	30%
Glucose	103.00	91.08	96.10
Fructose	91.65	91.12	101.98
Sucrose	94.48	90.14	91.21
Raffinose	90.89	102.38	103.05
Stachyose	94.86	97.88	92.37
Verbascose	98.15	94.44	91.57

3.3.3 Quantitative analyses

Quantitative analyses were performed building six calibration curves corresponding to glucose, fructose, sucrose, raffinose, stachyose and verbascose, in the ranges reported in Table 9. All coefficients of correlation are resulted equal or superior to 0.990.

Table 9.Calibration curves

Carbohydrates	Range(ppm)	Slopes	R^2
Glucose	2÷42	4.603	0.994
Fructose	2÷82	2.969	0.996
Sucrose	3÷83	0.833	0.992

Raffinose	2÷42	0.903	0.993
Stachyose	1÷81	0.616	0.994
Verbascose	1÷33	0.491	0.990

Sucrose are resulted the principal carbohydrate in leguminosae panel seeds, in according to Taira, 1990, followed by raffinose and stachyose; glucose, fructose and sucrose are in a small percentage, their sum is lower than 10% (Figure 5).

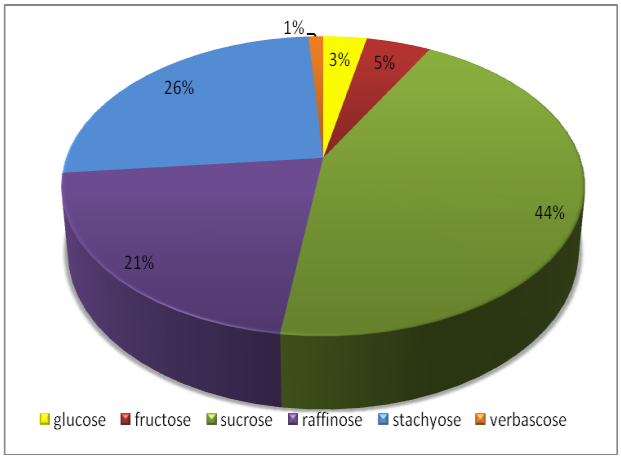

Figure 5. Carbohydrates composition of a leguminosae panel sample.

3.3.4 Optimization of sample extraction method

The conditions for extraction of carbohydrates from the panel of Leguminosae have been developed by optimizing the parameters related to water ratio: substrate, temperature and pH in order to get an extraction as exhaustive as possible. The choice of water as a solvent was necessary because the yeast cells could not tolerate the presence of organic solvents. Under optimum conditions (water: substrate 1:10 at a temperature of 70 ° C), repeating the extraction procedure on the residue 3 times, it was

obtained a quantity of carbohydrate in the extract of about 13% of the dry weight of panel of departure, which is close to the total oligosaccharides percentage.

A) Evaluation of extraction steps

Eight samples were analyzed corresponding to successive extraction steps. Obviously the carbohydrates content decreased progressively from the first extraction step, but in the seven and the eight only respectively 1.5% and 0.5% of carbohydrates are recovered (Figure 6). Considering the additional costs of industrial production for each step, the extraction process could be stop to the six step.

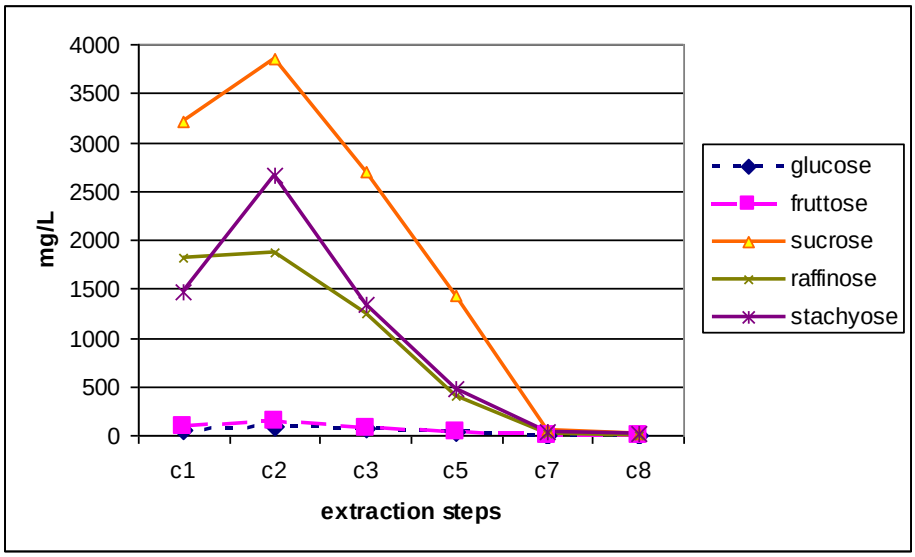

Figure 6-Carbohydrates quantities (mg/L) extracted during repeated extraction step

B) Extraction of samples at different pH values.

These analyses were conducted with the aim to both extract the maximum carbohydrates content without loss of nitrogen from the panels. Three samples were analyzed: one at neutral pH, one acidified first extraction and one acidified after extraction. The total content of carbohydrates was higher in the sample acidified first extraction, mainly for sucrose concentration, while verbascose concentration was

a little lower (Figure 7). Probably the acidification process lead to a partial hydrolysis of verbascose in simple sugars. Furthermore the sample acidified first extraction permits the precipitation of the protein fraction that remains in the residue. This is an advantage in view of a possible use of the residue from the extraction industries affected by nitrogen sources, such as feed mills.

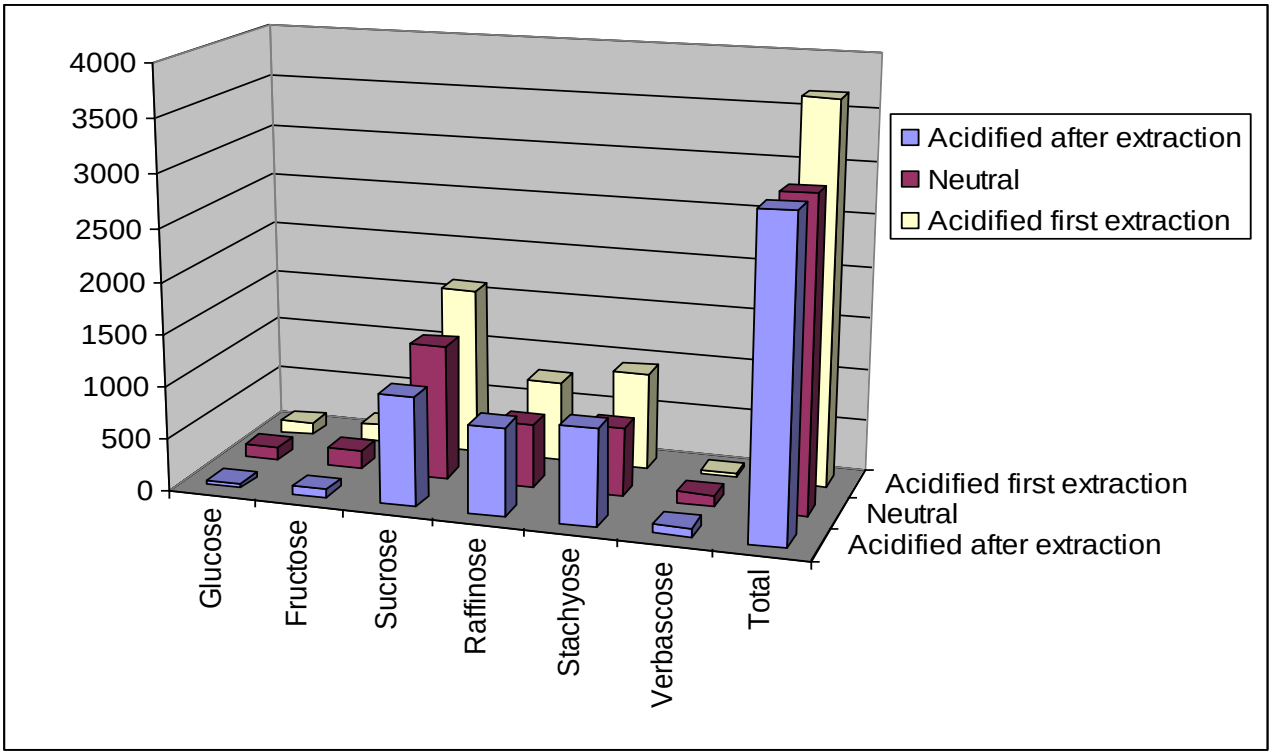

Figure 7-Results about carbohydrate extracted from leguminasae panels at different pH

3.3.5 Study on Saccharomyces cerevisiae cells metabolism

Study on metabolism of Saccharomyces cerevisiae cells was conducted following the procedure described in Materials and Method. In literature the fermentation of glucose, fructose, sucrose (by invertase), raffinose (by invertase and melibiase) is reported (Fraser, 1997) while there are not information about stachyose and verbascose.

In Figure 8, there are reported two chromatograms about carbohydrates composition: respectively the first one represents panels of leguminosae firstly of *Saccharomyces cerevisiae* inoculum and the second

one after 90 minutes of cells incubation. It is possible to observe that all initial carbohydrates are metabolized from yeast cells during the incubation time while two newly formed peaks are appeared.
The two unknown peaks showed in the chromatogram b, circled in the red colour, appeared immediately when *Saccharomyces cerevisiae* cells were incubated and their peak area increased in the first 10 minutes. This probably indicates that the two analytes are a product of *Saccharomyces cerevisiae* cells metabolism .The identification of these carbohydrates were performed by a ion-trap mass spectrometry analysis. Firstly the two unknown carbohydrates were collected after PAD detector, calculating the dead time injecting a blue dextran standard. Then samples were passed through a cationic membrane suppressor because HPLC mobile phases, sodium hydroxide and sodium acetate are not compatible with the ESI source. Finally mass spectrometry analysis (negative mode) had permitted to identify the two peaks as a disaccharide (m/z=341) and a trisaccharide (m/z=503), but no notices about the type of di and trisaccharides found are possible to obtain because isomers are not discriminated by an off-line mass spectrometry analysis.

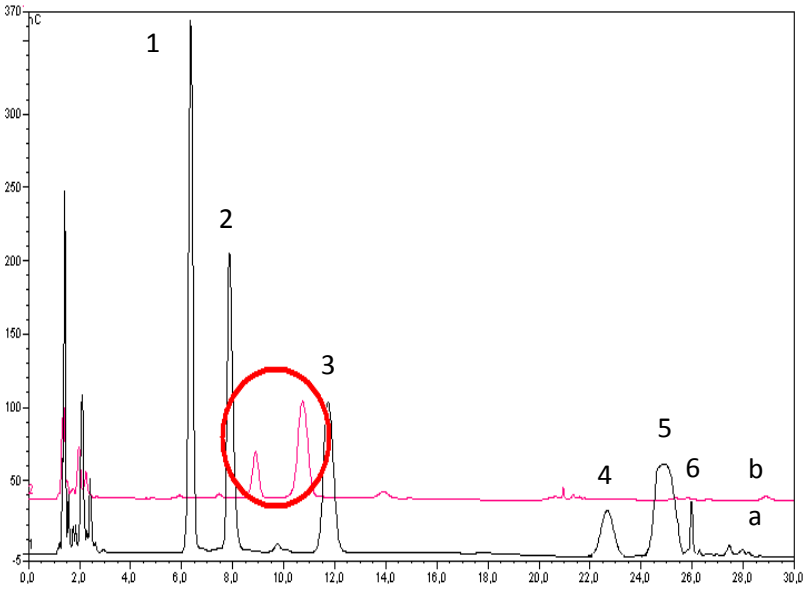

Figure 8-a-Chromatogram of the Leguminosae panel extract first of *Saccharomyces cerevisiae* inoculation; b-Chromatogram of the Leguminosae panel extract after 90 minutes of *Saccharomyces cerevisiae* incubation.

Regarding the others carbohydrates, it is possible to notice (Figure 9), in the first ten minutes of fermentation, an initial increase of glucose and fructose, and probably because extracellular invertase hydrolyzed sucrose in glucose and fructose. After 20 minutes also the sucrose concentration decreased and after 60 minutes all initials sugars are metabolized.

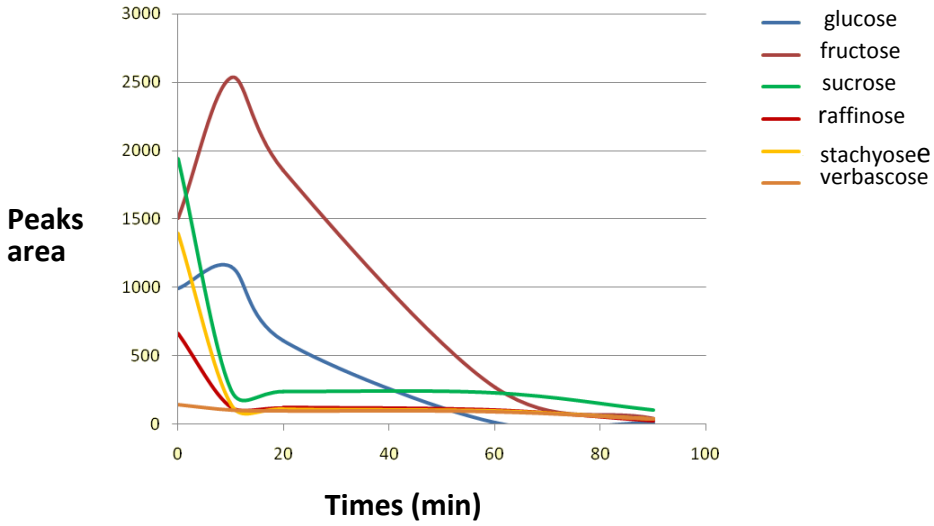

Figure 9- *Saccharomyces cerevisiae* cells metabolism during 90 minutes of incubation.

It was also tested a toxicity test (as indicator of the applicability of the substrate), consisting of controlling fermentation and monitoring CO_2 production by yeast cells placed in the substrate: the results indicate that the extract of leguminosae appears a good substrate for *Sacchromyces cerevisiae* cells.

3.4. Conclusions

The work represents an example of application of carbohydrates employed as process marker. The results pave the way for the possible use of panels of legume seeds defatted as a substrate for the

growth of strains of *Saccharomyces cerevisiae*. One advantage that may result from use of this substrate is certainly an economic nature, it is possible to derive the raw material at a low cost, since it is a waste product of refining processes. In addition, following the extraction of carbohydrates, growing weary, especially rich in nitrogen compounds could be further back on the market as a product for animal feed. Furthermore it would be a product with a great nutritional value because it took place the elimination of the sugar component, which is considered "anti-nutritional". One possible perspective of this work concerns the possibility of increasing the amount of carbohydrate to be extracted from the substrate through a preliminary hydrolysis, which also allows to extract sugars from soluble and insoluble fiber, composed of uronic acids, glucose, mannose and xylose.

3.5 Acknowledgements

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4. MALTULOSE, FUROSINE AND COLOUR INDICES AS PASTA DRYING PROCESS QUALITY MARKERS

4.1 Introduction

4.1.1 Pasta quality

Pasta is considered one of the foodstuff suited to a modern and balanced diet. Pasta is produced exclusively from durum wheat (*Triticum turgidum subs. durum*) semolina, salt and water semolina. In Italy pasta could only be made with 100% durum wheat products. (De Zorzi, et a, 2007), that the use of other undeclared cereals in pasta production constituted fraud.

Pasta is recognised as low in sodium and fat with no cholesterol and a rich source of complex carbohydrates (Giese, 1992), producing a low post-prandial response to glucose and insulin in the blood (Cleary, Brennan, 2006). However, it is low in protein and in essential amino acids, such as lysine and threonine .

The qualities that pasta must- have in order to meet the criteria and expectations of Italian consumers are as follows:

i. a uniform, amber-yellow colour without shades of grey o red;

ii. a clean surface appearance without brown, black or white spots or other signs indicating faulty milling;

iii. when cooked, pasta must not be glutinous on the surface i.e. stick together, but should have good ribbing and resistance to mastication;

iv. a pleasant aroma and taste typical to pasta;

v. practically zero contamination from chemical pesticides and preservatives.

Pasta quality is greatly affected by wheat protein content and quality: for example an high quality wheat must have a protein percentage/dry matter (Nx5.70) major than 15.0 and a gluten % /dry matter greater than 14.0 .Semolina pasta has this composition: an high carbohydrate content between 74-75%,

a proteins percentage of 10-12%, followed by water (11-12%) and fats (1-2%). (Landi, 1995) Semolina contains up to 80% starch and 2 to 3% nonstarch polysaccharides (Lintas, 1988).

Starch is important in determining cooking quality of pasta (Delcour et al, 2000) : as variations in starch properties impact water uptake, gel consistency, and gluten matrix integruty during cooking.

The amber-yellow colour of semolina is due to the presence in wheat of natural pigments from carotenoids particularly xantophylls and lutein (Borrelli et al, 1999).

4.1.2.Pasta processing

Pasta processing can be divided into four stages: mixing, kneading, shaping, and drying.

The goal of the mixing stage is to uniformly blend and properly hydrate ingredients. Semolina is typically hydrated to 30 -32% moisture content. The amount of water added will depend on the overall moisture content and the water binding properties of the various ingredients. For example, non starch polysaccharides have an high water binding capacity, which can affect water distribution in dough system during pasta processing and drying (Manthey and Schorno, 2002). Protein must be hydrated before gluten can form.

Pasta drying is a crucial operation for the quality of pasta, since modifications of main components can take place.

Shelf-life of pasta is commonly listed as two yeas. Typically, industry will dry pasta to 12% moisture, although the Federal Code of Regulations Food and Drug Admisnistration, 2003, allows the moisture content to be as high as 13%.

The pasta drying processes is generally divided into three main stages: pre-drying, final drying and cooling/stabilizing stages.

Initial pre-drying has the function to increase the rigidity of pasta to prevent pieces from sticking together.

The moisture content of the pasta entering the pre-dryer section of the dryer is 29-31%. The pre-drying stage removes about one-third of the total water in the pasta. The moisture content depends on the

temperature 18% (for ultrahigh temperature ≥ 80°C, drying) or 21 (for low temperature <60°C, drying).

During final dryer the product will have 18 to 21% moisture content upon entering the final dryer is critical:if drying is too fast the stresses near the surface of the product will exceed the strenght of pasta.

The product is brought to near ambient temperature and exposed to 50% relative humidity during the cooling/stabilization stage.

Advances in drying technology have resulted in three drying intensity categories that describe processing temperature and relative humidity ranges: conventional drying, high temperature drying and ultrahigh temperature drying (Manthey and Twombly, 2006) .

The traditional methods for drying pasta use low temperatures, LT (29–40 °C) and longer treatment times, Lt (24–60 h, LT–Lt), imitating the conditions that occur in the Mediterranean region.

High Temperature-Short Time (HT-ST) drying processes (using temperatures of around 75 ±3 °C) were introduced into commercial drying lines in 1974. Over the past few years, changes in pasta technology have increased the drying temperature from 75 ±3° C to 100 ±3° C and above (VHT-ST), reducing the drying time from the original 48 h to 2-3 h.

The main advantages deriving from the HT-ST and VHT-ST applications, are related to the reduction of microbial contamination and to the improvement of cooking quality of pasta. On the other hand, changes in the essential amino acid pattern and the reduction of amino acid bioavailability have been evidenced and related to the application of high drying temperatures. It is in fact well known that the drying process represents the most delicate step for these products because the Maillard reaction products are influenced by many factors such as temperature, water activity and chemical characteristics of the food system (Acquistucci, 2000).

4.1.3 Maillard reaction in pasta

The Maillard reaction (MR) occurs when carbonyl groups, usually from reducing sugars, condense with free amino groups from amino acids, peptides and proteins.

The reducing sugars are provided by damaged starch that can be a result of growing condition, milling, mixing with water or extraction. Therefore amino acid is generally from a lysine residue in protein.

Maillard reaction can occur during high temperature and ultrahigh temperature drying water activity of 0.75 and 0.80 and/or a moisture content of 15% are optimum moisture conditions for the Maillard reactions occurs in pasta (Manthey and Twombly, 2006) **.**

Water activity appears to be particularly important ; the initial reaction which produces glycosylamine plus water can be slowed by high *a* values. The subsequent browning stages may be inhibited by the production of three moles of water for each mole of glucose consumed (Sensidoni et al, 1999).

There are three stages to the MR: early reaction; advanced reaction, and final MR. The first corresponds to the steps without browning, the second to the reactions leading to volatile or soluble substances and the third to the reactions leading to insoluble brown polymers (Mauron, 1981)

In the first step, the carbonyl group of the reducing carbohydrate and the free amino group of the amino acid or protein form a condensation product with the loss of a molecule of water to form a *Schiff* base.

The *N*-substituted glycosylamine derived from the cyclisation of the *Schiff* base is converted to the 1-amino-1-deoxy-2-ketose by the Amadori rearrangement. The MR is much slower at this stage if the process temperature is below a certain value. The early stages of MR can reduce nutrition value, because they induce a decrease in amino acid availability. For example, the ε-amino group of lysine forms a stable Amadori compound (*ε-fructosyl-lysine*) which blocks the amino acid; *ε fructosyl-lysine* is converted by acid hydrolysis into furosine.

The subsequent browning stages may be inhibited by the production of three moles of water for each mole of glucose consumed. In addition, water may enhance deamination reactions for the production of furfural or hydroxyl-methyl-furfural (HMF) (Sensidoni et al, 1999).

In literature some works (Anese et al, 1999, García-Baños, 2004) reported furosine as a good an index of the early stages of MR.

The determination of reaction products, such as *ε-fructosyl-lysine* by furosine determination (Resmini et al, 1990; Pagani et al, 1992) is also unsatisfactory because they are degraded during drastic heat treatment. (Sensidoni et al, 1999)

Therefore for the second stage of MR *2-acetyl-3-D-glucopyranosylfuran* (AGPF) have been proposed as markers (Resmini et al, 1993) **.**

Aquistucci, 2000, found that small amounts of furosine formed during milling and did not increase during extrusion while at the end of the drying cycle concentration an higher content of furosine was determined. The LT process did not cause any change in furosine concentration, while the formation of this early Maillard reaction compound was found to be remarkable for the HT and HTc processes. In these last cases, because of the progressive consumption of furosine to form advanced MRPs, its concentration reached a maximum and then decreased.

Intermediate and advanced Maillard reaction products if, on the one hand, they are reported to have antioxidant and anti-mutagenic properties (Usman, and Hosono, 1997; Yen et al, 1993), on the other hand some reports deal with their pro-oxidant as well as mutagenic properties (Pischetsrieder et al, 1998, Anese et al, 1999)

Regarding changes in pasta colour, no variations during LT processes were measured. On the contrary, in HT processes the increase in the chain breaking activity in the last stages of the drying process corresponded to a slight increase in hue angle values. This behaviour, consisting of a reduction in the antiradical properties of pasta in the early stages of the drying process and an increase in the later ones, resulted more strongly for pasta dried at 110°C (HTc process) (Anese et al, 1999) . Some authors reported colour as marker of MR, regarding yellow index (b*), red index (a*) and lightness index (L*) **(**Anese et al, 1999, Acquistucci, 1999, Sensidoni et al, 1999).

The yellow index is an aesthetic indicator which is quite important in the pasta food market. The yellow colour in pasta depends on natural pigments in semolina, such as xanthophylls and carotenoids, and enzymatic activity, especially polyphenol oxidase and peroxidase.

The yellow index (b*) demonstrated good retention of pigment depending on enzymatic inactivation. This colour index for pasta systems is thus insufficient for monitoring the kinetics of the complex non enzymatic browning reaction, due to interference from other compounds. (Sensidoni et al, 1999).

The red index is strictly related to the development of the MR (Oliver et al, 1993).

Aquistucci, 2000, showed that the three colorimetric indices varied during the transformation of semolina into dough. The lightness index (L*) decreased during the process with the consequent increase of the brown index whereas the other indices increased because of the temperature applied while the red index (a*) increased.

4.1.4 Carbohydrates composition of pasta

There are not regulations for labelling the net carbohydrates or bio available carbohydrates in foods like pasta.

Net carbohydrates was calculated by Lilla et al, 2005 as: (100-protein-fat-water-ash-sugar alcohols-fiber- polydestrose- glycerin- fructan.) Net carbohydrates was reported to have a value of 36.9% in pasta.(total nonsoluble fiber).

Starch is present in a concentration of 73.5g/100g of pasta, while total sugars 6.7%, which maltose is the principal carbohydrate with a concentration of 6.1 g/100g of pasta (Widdowson et al, 2004) .

Pasta processing can produce changes in carbohydrate content, thus during mixing, extruding and drying phases, starch can suffer damage, releasing free maltose. The changes of free carbohydrates (maltose, glucose and fructose) during drying of different pasta products have been previously studied (Lintas and D'Appolonia, 1973, Resmini and Pellegrino, 2004, Sensidoni et al, 1996).

Garcìa-Baños et al, 2004 detected maltulose in dried pasta samples by Gas Chromatography analysis. Maltulose is an epimerization product of maltose, a reducing carbohydrate which could participate to Maillard reaction; although the Maillard reaction and maltose isomerization proceed at different rates, both reactions may occur simultaneously at the high temperatures achieved during the manufacture of cereal products (Rada-Mendozal et al, 2004).

4.1.5 Aims of this work

Parallel analyses on furosine content, colours index and carbohydrates composition are conducted on pasta samples purchased by a an Italian pasta factory in order to correlate these parameters with drying pasta stage and in relationship with pasta shapes.

The same analysis were performed also on relative semolina samples with the aim to verify if furosine index, red, colour parameters, reducing sugars and malulose changed in relationship with Maillard reaction and, therefore, if they could be proposed as pasta processing markers.

Finally this model was tested on same commercials pasta samples of different quality, conducing a Principal Component Analysis.

4.2 Materials and Method

4.2.1 Samples of pasta analyzed

Ten dried pasta samples (six samples of spaghetti, one sample of penne and four samples of farfalle) and their relative semolina, produced for our study by an Italian pasta factory (Cellino, Santa Giusta, Oristano), were analysed. Furthermore, seven commercial samples (one sample of spaghetti, three of penne and three of farfalle) from different manufacturers, purchased at local markets, were considered in this study.

4.2.2 Pasta drying

Pasta samples were dried in an industrial plant (Bühler). Each pasta shape was subjected to a different combination of temperature and time. All drying cycles consisted of multistep processes: for penne and farfalle temperature was high at the initial phase of the drying and then was decreased in the final stages of the process, while for spaghetti temperature was increased with time increasing (Figure 1).
Total drying time deeply varied depending on pasta shape: spaghetti needed the highest drying time, (almost 300 minutes), while farfalle the lowest, about 205 minutes, finally penne shape was between spaghetti and farfalle (240 minutes).

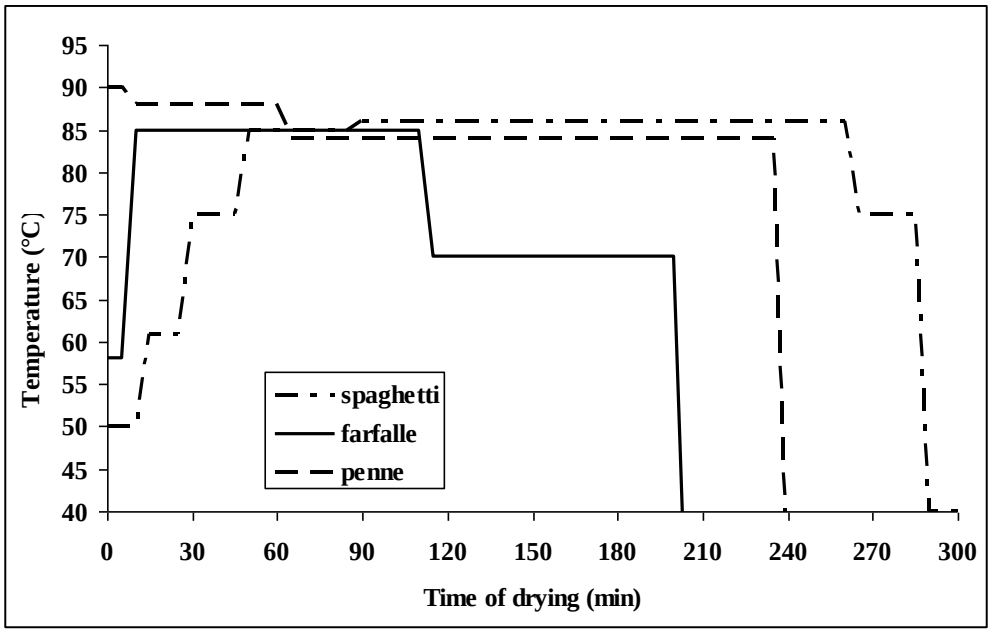

Figure 1-Drying cycles for each pasta format

4.2.3 Heat damage evaluation

In order to easily compare different drying cycles, depending on pasta size, the degree of heat damage of each sample was expressed in terms of cook value $C^{z}_{T_{ref}}$. The cook value was obtained from the integration of the time-temperature history during the drying process and expressed in minutes:

$$C^{z}_{T_{ref}} = \int_{0}^{t} 10^{(T-T_{ref})/z}\, dt$$

where:

t = time [min]

T_{ref} = reference temperature; set equal to 100 °C

z = temperature increase that induces a 10 fold increase of the reaction rate of the chemical reaction taken as reference; z was set at 22.9 ^{o}C for the furosine production kinetic, according to the value reported by Zardetto, 2003 and at 33 ^{o}C for the standard cooking rate calculation, as already reported for cooking textural and colour modification (Holdsworth, 1985) Three determinations were performed for each pasta shape.

4.2.4 Furosine determination

The determination of furosine in pasta and semolina samples was performed following the method proposed by Resmini et al., 1990 and slightly modified as follows: 200 mg of samples was weighed (corresponding to approximately 20 mg of protein) and hydrolysed with 10 mL of HCl 8N, under inert conditions at 110 ° C for 23 h in a screw-capped Pyrex vial with PTFE-faced septa. After hydrolysis, the sample was filtered with a 0.45 µm PTFE membrane and led to a volume of 10 mL with water to keep the dilution ratios, thereby offsetting the possible evaporation during the hydrolysis.

HPLC analysis was performed by using a liquid chromatograph PerkinElmer 200 IC pump series (PerkinElmer Waltham, Ma, USA), equipped with a mixing system with four channels and coupled with a UV-visible detector Hewlett Packard 1050 series (Agilent Technologies, Palo Alto, CA, USA) set at a wavelength of 280 nm.

The samples were manually injected into the chromatographic column using a Rheodyne® injector, Model 7125, with loop of 20 µL. The column used was a Luna 5u C18 (2 x 250 mm, 5 µm, Phenomenex Inc., CA, USA), preceded by a pre-column C18 (Phenomenex). Acquisition and processing of data were obtained by DAN-Client software; results were expressed as mg/100 g of protein. All the analyses were carried out in duplicate. Proteins (Nx5,70) content was determined according to the AOAC procedures (AOAC 1995).

4.2.5 Carbohydrates determination

Samples prepation proposed by Garcìa-Baños, J, L, 2004 for the extraction of maltulose from pasta samples using 80% ethanol was compared with the following method: two grams of samples (pasta or semolina) were weighed and 50 mL of HPLC grade water was added and homogenized with Turrax, then the solution was heated at 80°C for 60 minutes under continuous stirring andcentrifuged for 30 minutes at 8500 rpm. After filtration through a 0.22 μm membrane filter (Analytical Technology, Cernusco sul Naviglio, Milano, Italia), samples were injected into HPLC by an autosampler AS50 Dionex.

Elution of carbohydrates was performed at room temperature on a Dionex Carbopac PA20 (3X150mm), equipped with pre-column Carbopac PA 20 CarboPac PA20(3x150 mm) at a constant flow of 0,5 ml/min. Gradient elution was applied using three solvent: water (eluent A), 0.6 M aqueous sodium hydroxide (eluent B) and 0.5 M sodium acetate solution (eluent C). All mobile phases were sparged and pressurized with helium to prevent adsorption of atmospheric carbon dioxide and subsequent production of carbonate, which would act as displacing ion and shorten retention time.

Standards of galactose, glucose, fructose, sucrose, maltulose, maltose, raffinose, stachyose, verbascose and maltodextrins standards were purchased from Sigma Aldrich. All stock solutions were prepared at 5 $mgmL^{-1}$ with HPLC-grade water and filtered on a 0.22 μm membrane filter.

4.2.6. Colour determination

Colour determination were conducted both by a Minolta Colorimeter (CM 2600d, Minolta Co., Osaka Japan) equipped with a standard illuminant D65 both, a more traditional employed method (Anese, 1999, Sensidoni, 1999 Acquistucci, 2000) and by image analysis.

Colour determinations by colorimeter were carried out on L* (lightness, black = 0, white = 100) index, a* (redness, a* > 0, green < 0) and b* (yellowness, b* > 0, blue < 0) index of both semolina and pasta samples, quantifying on each sample using a 2nd position of the standard observer. The instrument was calibrated before each analysis with white and black standard tiles. A total of 10 determinations were performed for each pasta sample.

Regarding image analysis semolina and pasta samples were scanned by means a desktop flatbed scanner (Hewlett Packard Scanjet 8200, Palo Alto, CA, USA) at 236 pixels per cm (600 dpi of resolution; true colour – 24 bit), equipped with a cold cathode lamp for reflective scanning. All images were scanned at the same conditions, by positioning on the scanner 10 pasta samples: during image acquisition, the scanner was held in a black box, in order to exclude surrounding light and external reflections. Flatbed scanner colour was characterized and corrected as previously reported (Romani et al, 2009) .

4.2.7. Chemometric analysis

Principal component analysis (PCA) was performed on by means of the STATGRAPHIC Centurion Version XV (U.S) on both commercial and Cellino samples.

4.3. Results and discussion

4.3.1 Determination of Furosine index

The method reported in Materials and Method had permitted a complete separation of furosine in a retention time of about 6 minutes. The recognition of furosine was done comparing the retention time of analyte in sample with the same in standard solution (Figure 2).

In semolina samples furosine resulted present with HPLC-UV analysis; to verify if this was a correct assumption, an HPLC system (Waters.) was used interfaced to a time of flight mass spectrometer (Q-

TOF) (Micromass). The mass spectra obtained (relative to a standard, a sample of dried pasta and a semolina sample) showed that the furosine (m / z = 255) was not present in the semolina and the contaminant was only present in semolina sample (Figure 3).

The false positive detected by UV/vis is probably due to the presence of a compound with characteristics of polarity and UV absorption very similar to our analyte , but of structure and different molecular weight.

The HPLC-UV method was validated in terms of limit of detection (LOD) and limit of quantification (LOQ), linearity, precision and recovery, according to EURACHEM guidelines on blank.

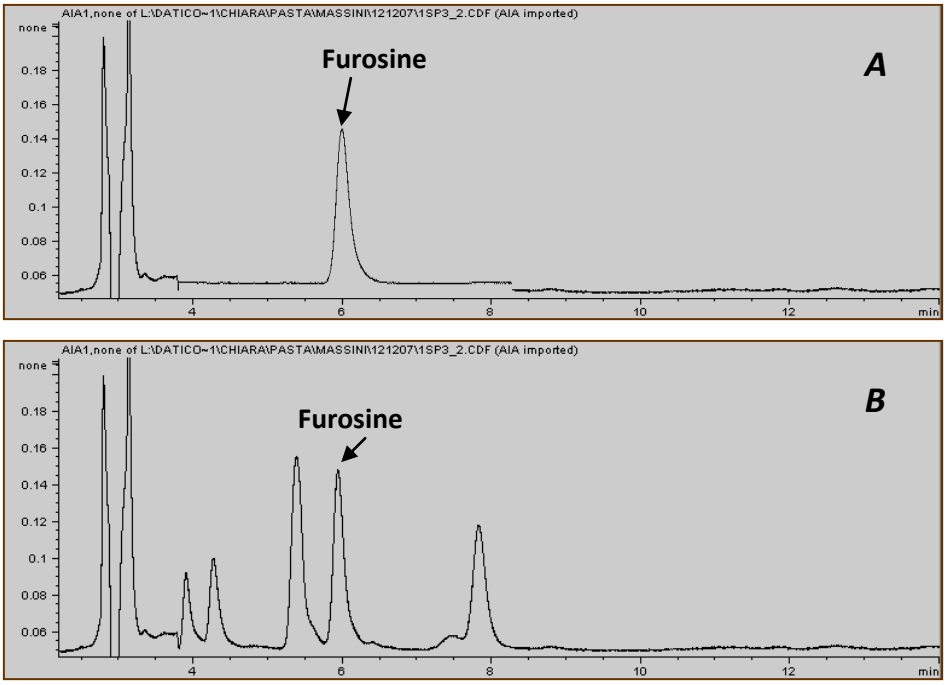

Figure 2. Furosine standard chromatogram and pasta sample.

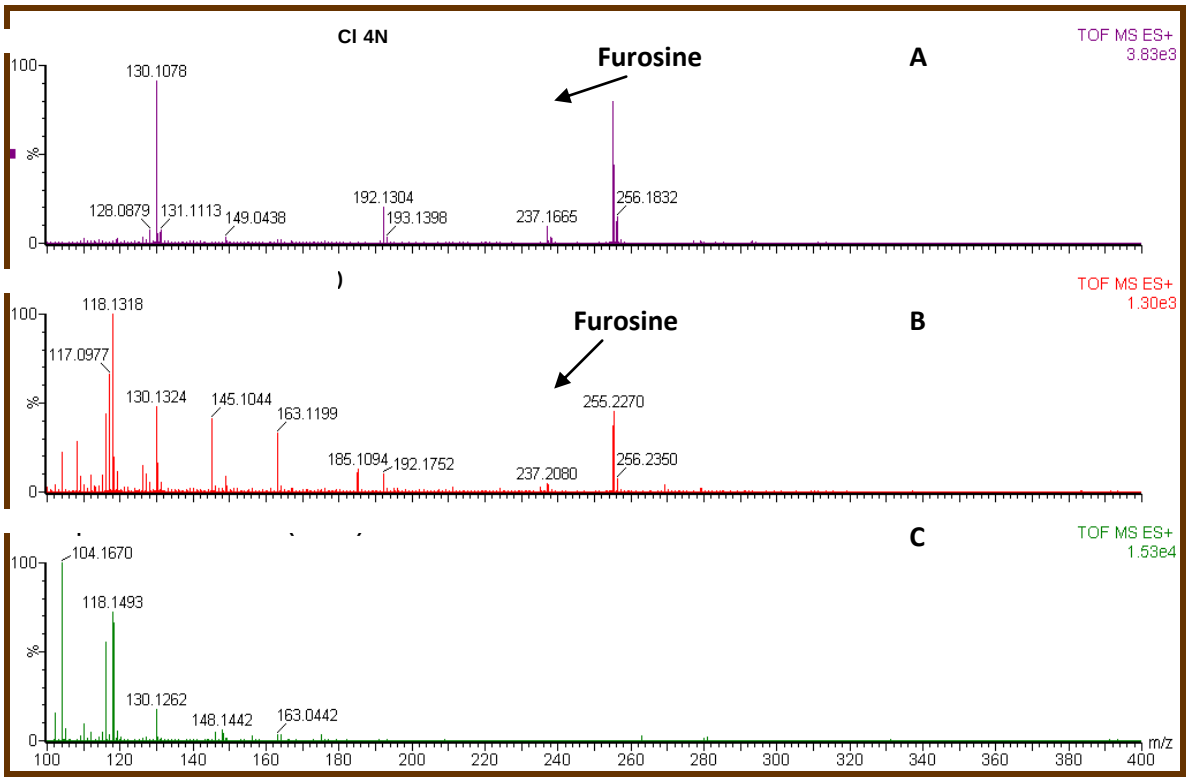

Figure 3. Mass spectra obtained by HPLC-Q-TOF analysis. A-Furosine standard mass spectrum; B-Pasta sample mass spectrum; C-Semolina mass spectrum.

Limit of detection, calculated as $y_D = \mu_B + 2t_{(95\%;\ n-1)}\sigma_b$, (where μ_B is the mean value of ten measurement of blanks, σ_b is the standard deviation of blank and t is a constant of the *t*- Student distribution) was 0.22 ppm , while limit of quantitation , that was determinate by formula $yQ = \mu B + 10\sigma_b$, was 0.27ppm.

Linearity was established at seven different concentration levels (0,5-50ppm) and three replicated injections were performed at each level, with R^2=0.999.

Precision was evaluated at two concentration levels (5ppm- 22.5ppm), in terms of intra-day (n=7), and inter-day repeatability (n=14) of area, providing RSD% values <0,7%.

The last parameter, recovery, was determined fortifying a pasta sample with furosine standard, at three levels of percentage (15, 30, 45%). A good recovery of method was found, with recovery percentage in a range of 102-104%.

Furosine was quantified by external standard method building a calibration curve, using semolina as matrix. Then, data obtained were compared with protein content of each samples, determined by Kjeldahl method and using 5.70 as conversion factor for the total nitrogen. In Table 1 data about furosine content in Cellino samples are reported.

Table 1. Furosine values of Cellino samples.

SAMPLES	Protein content (%) Mean value ±Sd	Furosine (mg/100 g pasta) Mean value ±Sd	Furosine (mg/100 g protein) Mean value ±Sd
Spaghetti (n=5)	11.41 ± 0.07	70.47 ± 0.97	617.59 ± 4.90
Penne rigate (n=1)	11.54 ± 0.27	55.90 ± 6.28	483.63 ± 43.28
Farfalle (n=3)	11.82 ± 0.10	35.36 ± 1.80	299.17 ± 12.76

Spaghetti showed the highest content of furosine (mean value: 618 mg/100g protein) , followed by penne and farfalle.

Probably these difference between formats could be attributed to the fact that, for each format is provided, at the same temperature of treatment, a suitable drying time, which leads to degradation in terms of Lysine "locked", proportional to the processing itself.

With the aim to verify if this assumption was correct also for commercials samples, penne and spaghetti of different trade-marks and prices were analysed.

In commercial samples we don't know anything about pasta drying step, although this, spaghetti samples showed an higher furosine index than relative trademarks penne. Differences between A, B and C samples could be due both to different drying process or semolina quality (Figure 4).

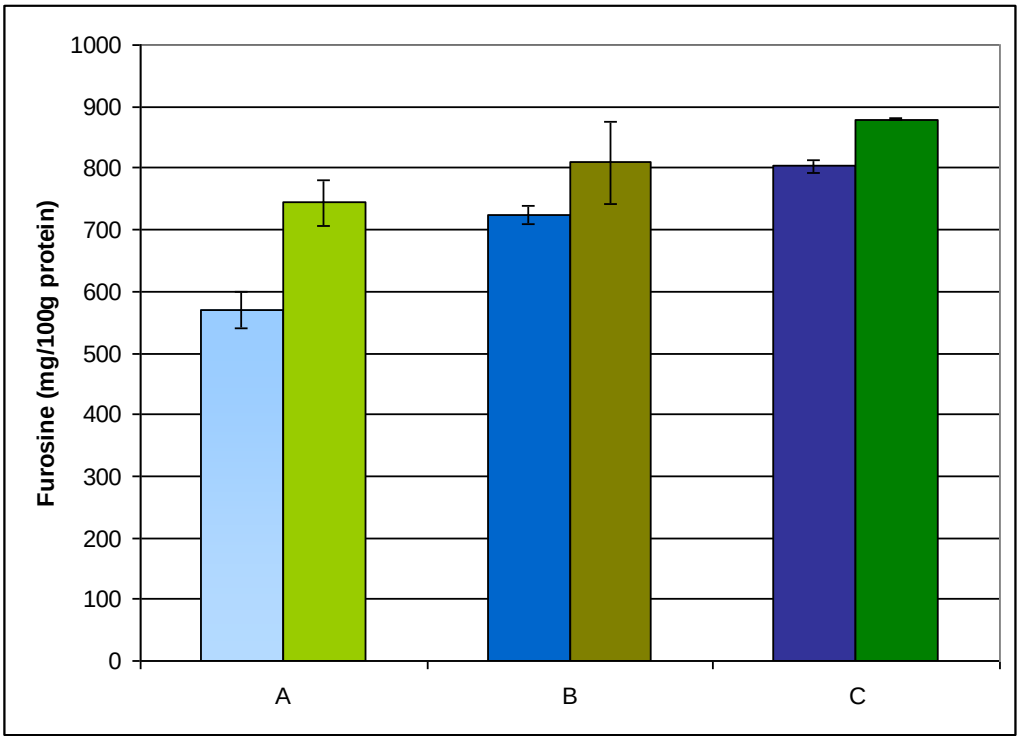

Figure 4. Quantitative of furosine (mg/100g protein) on pasta commercial samples. In blue scale: penne format, in green scale: spaghetti format. A, B, C are samples of three different trademarks of growing price.

Furosine index could be considered a good marker in relationship to the early step of Maillard reaction, however it is degraded during drastic heat treatment (Sensidoni et al,1999) and in the advanced MR step other products could be formed. A low content of furosine could mask an higher temperature and time drying step.

Therefore others analyses on semolina and pasta are conducted: colour analysis and carbohydrates analyses.

4.3.2 Colour determination

Great differences for cook effect values were found between considered shapes depending on drying cycle conditions (Table 2).

Table 2.Values of colour indices

	L*semolina	L*pasta	a*semolina	a* pasta	b*semolina	b*pasta	$C^{22.9}_{100}$	C_0
farfalle								
AP8	90.4 ± 0.2a	38.7 ± 3.2a	1.1 ± 0.1b	3.8 ± 1.2a	18.3 ± 0.5c	26.3 ± 5.3b	12.8 ± 0.4	28.4 ± 0.9
BA8	89.1 ± 0.4b	43.1 ± 5.9a	1.5 ± 0.1a	4.9 ± 1.6a	22.3 ± 0.9a	31.1 ± 4.8a	12.1 ± 0.3	27.4 ± 1.6
CZ7	89.4 ± 0.4b	40.0 ± 5.3a	1.4 ± 0.1a	1.3 ± 0.6b	20.4 ± 0.9b	19.8 ± 0.9c	11.7 ± 0.3	27.8 ± 1.0
spaghetti								
AS8	89.9 ± 0.6b	60.9 ± 0.5c	1.2 ± 0.1b	6.4 ± 0.3b	19.9 ± 0.8c	42.6 ± 1.3b	27.0 ± 1.2	53.9 ± 1.4
AP8	90.4 ± 0.2a	62.2 ± 0.3a	1.1 ± 0.1b	5.5 ± 0.1c	18.3 ± 0.5d	42.4 ± 0.3b	27.1 ± 1.0	54.2 ± 1.1
CH7	89.8 ± 0.4b	57.0 ± 0.4d	1.2 ± 0.1b	5.3 ± 0.2c	20.9 ± 0.4b	42.5 ± 0.6b	26.8 ± 0.9	53.4 ± 1.5
DG7	89.0 ± 0.5c	55.1 ± 0.3f	1.4 ± 0.1a	6.2 ± 0.2b	21.4 ± 0.6b	40.3 ± 0.6c	27.4 ± 1.1	53.9 ± 1.3

DM7	90.4 ± 0.5a	56.6 ± 0.4d	1.2 ± 0.2b	5.1 ± 0.3c	21.2 ± 0.4b	43.3 ± 0.5b	29.1 ± 1.1	57.9 ± 1.3
BA8	89.1 ± 0.4c	61.3 ± 0.4b	1.5 ± 0.1a	7.2 ± 0.2a	22.3 ± 0.1a	47.5 ± 0.5a	27.1 ± 1.0	54.2 ± 1.7

[A] Data are expressed as mean ± standard deviations of ten determinations. Means within each column with different letters significantly differ ($P<0.05$)

Semolina and pasta parameters both from reflectance colorimeter and image analysis are reported in Table 3. Among semolina samples for farfalle production, AP8 presented a significantly highest value for L* parameter and on the contrary the lowest a* one. With regard to b* parameter, BA8 sample shown the highest value and AP8 the lowest one. Among farfalle samples, after the drying process L* parameter considerably decreased as expected and no significant differences were found. On the other hand, a* values slightly increased: AP8 and BA8 presented the highest a* values. BA8 sample that was produced form the semolina with the highest b* values shown the highest b* values also after the drying process.

Among semolina samples used for spaghetti manufacturing, AP8 presented the highest L* values while DM7 the lowest ones; same results were obtained for spaghetti L* parameters.

With regard to a* parameter, BA8 and DG7 semolina and spaghetti samples shown the highest values. Finally, BA8 semolina and spaghetti sample presented also the highest b* values.

Direct correlation was found between pasta colour parameters and drying cycle both expressed as $C^{22.9}{}_{100}$ and C_0, as previously reported by Massini et al. (1999).

Colour parameters of commercial pasta shapes were in the range of produced samples.

Table 3. Reflectance colorimeter and image analysis

	Spectrophotometer			Image analysis		
	L*	**a***	**b***	**L***	**a***	**b***
farfalle						
AP8	38.7 ± 3.2	3.8 ± 1.2	26.3 ± 5.3	49.4 ± 0.5	-7.7 ± 0.3	48.8 ± 1.3
BA8	43.1 ± 5.9	4.9 ± 1.6	31.1 ± 4.8	53.6 ± 1.0	-3.6 ± 0.4	49.4 ± 2.4
CZ7	40.0 ± 5.3	1.3 ± 0.6	19.8 ± 0.9	52.7 ± 0.9	-7.5 ± 0.3	37.0 ± 1.3
barilla	41.0± 2.5	1.6 ± 0.3	20.8 ± 0.3	52.5 ± 0.9	-7.7 ± 0.3	39.2 ± 1.8
garofalo	32.0 ± 9.4	1.9 ± 0.9	19.5 ± 0.7	49.4 ± 0.5	-8.2 ± 0.3	37.1 ± 1.6
conad	44.1 ± 4.4	1.2 ± 0.2	17.7 ± 1.0	55.7 ± 1.0	-6.1 ± 0.3	34.9 ± 0.9
spaghetti						
AS8	60.9 ± 0.5	6.4 ± 0.3	42.6 ± 1.3	74.1 ± 0.8	-16.1 ± 0.3	67.9 ± 1.3
AP8	62.2 ± 0.3	5.5 ± 0.1	42.4 ± 0.3	76.0 ± 0.5	-17.6 ± 0.3	67.3 ± 0.9
CH7	61.7 ± 0.4	5.3 ± 0.2	42.5 ± 0.6	72.4 ± 0.4	-3.9 ± 0.3	71.2 ± 0.8
DG7	59.2 ± 0.3	6.2 ± 0.2	40.3 ± 0.6	69.4 ± 0.6	1.34 ± 0.2	69.0 ± 1.2
DM7	61.4 ± 0.4	5.1 ± 0.3	43.3 ± 0.5	71.9 ± 0.5	0.0 ± 0.6	70.5 ± 0.9
BA8	61.3 ± 0.4	7.2 ± 0.2	47.5 ± 0.5	73.8 ± 1.0	-6.2 ± 0.4	72.1 ± 1.1
barilla	57.4 ± 0.5	5.5 ± 0.1	39.5 ± 0.6	69.6 ± 1.0	-2.7 ± 0.4	65.0 ± 1.4
penne						
DA7	46.7 ± 0.2	0.8 ± 0.1	21.7 ± 0.5	59.5 ± 1.8	-6.3 ± 0.4	40.5 ± 1.4
conad	46.0 ± 0.7	1.5 ± 0.1	21.0 ± 0.5	57.6 ± 1.0	-7.5 ± 0.2	39.4 ± 1.2
divella	44.9 ± 1.2	1.4 ± 0.1	19.7 ± 0.7	56.5 ± 0.9	-6.3 ± 0.3	37.6 ± 1.4
barilla	47.0 ± 1.1	1.2 ± 0.1	22.1 ± 0.4	58.6 ± 0.8	-6.7 ± 0.4	40.9 ± 1.6

[A] Data are expressed as mean ± standard deviations of ten determinations. Means within each column with different letters significantly differ ($p<0.05$).

4.3.3 Carbohydrates analysis

Few works are reported in literature about carbohydrates analyses in pasta, by thin-layer chromatography, enzymatic assay (Lintas and D'Appolonia, 1973), gas chromatography techniques (García-Baños, 2004) .No works are reported about pasta carbohydrates composition by HPAEC-PAD: In this study, HPAEC-PAD conditions of analysis have been optimized, in terms of choice of column and gradient of elution with the aim of separating both simples and complex sugars as maltodextrins.

Elution conditions having the suitable selectivity have been obtained by developing a first isocratic step where sodium hydroxide concentration was maintained constant at 18mM for 15 minutes, followed by a first gradient step where sodium hydroxide was increased to 60mM and sodium acetate from 0 to 15mM in 10 minutes and a second gradient step increasing sodium hydroxide to 150 mM and sodium hydroxide concentration to 125 mM. in 25 minutes. Each analysis was followed by a washing step and by a conditioning step where mobile phase compositions was maintained at initial conditions for 40 minutes.

The two methods of sample extraction described in Material and Methods gave the same results in terms of chromatographic profiles and recovery, therefore we chose to extract samples with water heated at 80°C.

The developed method permitted a complete separation of all carbohydrate present in both durum wheat semolina and in pasta samples.

Carbohydrates identification was performed by comparing retention times of samples with commercial standards and by adding small amounts of standards samples to confirm peak identity. Elution order was the following: monosaccharides galactose, glucose and fructose, disaccharides sucrose, maltulose, trisaccharide raffinose, tetrasaccharide stachyose , pentasaccharide verbascose, maltose and maltodextrins, as reported in Figure 5. Raffinose, stachyose, verbascose are named of "raffinose family", they are normally distributed in leguminous but also in whole grains and are known for their

negative effect of flatulence although interesting prebiotics properties have been described from them (Matteuzzi et al, 2004).

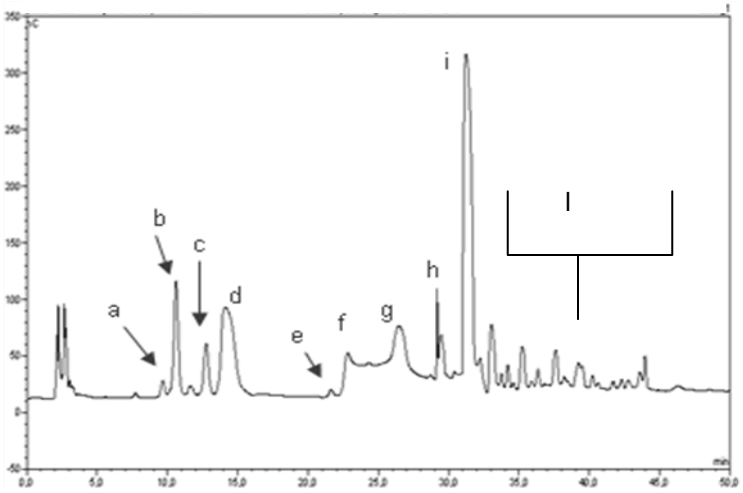

Figure 5- Chromatograms of a pasta samples a-galactose; b-glucose; c-fructose; d-sucrose, e-maltulose; f-raffinose; g-stachyose; h-verbascose; i-maltose; l-maltodextrins

The HPAEC-PAD method was validated following EURACHEM guidelines on standards of galactose, glucose, fructose, sucrose, maltulose, raffinose, stachyose, verbascose and maltose.

Limits of detection and quantitation were in the range, respectively, from 0.05 to 7.03 ppm and from 0,09 to 13,53 ppm. In particular LOD of maltulose was 0.28 ppm and LOQ 0.51 ppm.

Linearity was established from each standard at five different equispaced concentration levels and three replicated injections were performed at each level: galactose (b=0.785) in 9÷-60.5 mg/L range , glucose (b=1.016) and fructose (b=1.381): 1.5÷141,5mg/L, sucrose (b=0.523): 2÷202 mg/L, maltulose (b=1.273), raffinose (0.850), stachyose (0.715), verbascose (0.336) :1÷121 mg/L, maltose (0.353): 10÷1210 mg/L. Good linearity was established in the range analyzed with R^2=0,990-0,999.

Precision was evaluated in terms of intra-day and inter-day repeatability of area, providing RSD% values respectively in the 1.76-4.83 range and in the 2.88-5.24 range

With the purpose of determine recovery, samples were fortified with two standards, maltose and maltulose at three different concentrations; obtained values were in the range between 92.4% and 97.5%.

As well known, reducing carbohydrates are involved in Maillard reaction , therefore we have evaluated the eventual changes in carbohydrates distribution comparing semolina and relative pasta samples composition.

In semolina samples maltose is the principal reducing carbohydrate (88% of total sugar) followed by glucose (3%), fructose (2%), and galactose.

Changes in carbohydrates composition were observed, with a generally decrease in reducing sugars (Table 4) mainly regarding maltose content in all shapes of pasta considered (Figure 6) : its mean percentage value was 72 (16% lower than in semolina sample).

Regarding penne and spaghetti commercial samples, a similar carbohydrates distribution was observed, as reported in Table 5.

Maltulose was detected in all pasta samples, as previously reported by (García-Baños, 2004).

Considering that on one hand maltulose is a product of isomerization of maltose and on the other hand it is a reducing carbohydrate that could participate to the Maillard reaction, we have investigated if maltulose could be considered a good marker of pasta drying process.Therefore, it was verified the presence of those disaccharides in semolina samples. Analyses were conducted both on semolina relative to Cellino pasta samples and on some semolina stored during 30 days.

Despite the fact that in previous works maltulose was not detected in fresh pasta samples by GC technique, and therefore we could expected to find it only in dried pasta samples, we detected maltulose by HPAEC-PAD also in all semolina Cellino samples, with a concentration higher than limit of quantitation.

We can hypothesize that maltulose presence in semolina samples could be related to storage conditions in terms of temperature and it could also be influenced by mill process. To confirm this idea we analyzed a semolina sample (produced by Agiugaro &Figna) , the first one-time grinded and the second two-time grinded. Maltulose concentration of this was about two-fold (8.01±0.19 mg/100g semolina respect than 4.37 ±0.11 mg/100g semolina).

Maltulose analysis was also conducted on typical Calabria fresh samples named "Fileja" (n=3).Results showed values in the range 9.04÷13.24 mg/100g fresh pasta, in disagreement with García-Baños, 2004 analyses on fresh pasta samples with different shapes.

Probably maltulose alone it could not be considered as a good marker of pasta quality, due to its presence in semolina and in fresh pasta samples. Perhaps, considering the relative differences between pasta and relative semolina, maltulose concentration could give a possible indication about drying pasta step conditions, although many notices about semolina quality and storage would be necessary.

Table 4. Reducing sugars in pasta and relative semolina samples.

CARBOHYDRATES (mg/100g)	FARFALLE (n=3)	SEMOLINA FARFALLE (n=3)	PENNE RIGATE (n=1)	SEMOLINA PENNE RIGATE (n=1)	SPAGHETTI (n=5)	SEMOLINA SPAGHETTI (n=5)
Galactose (RSD%)	0.42÷3.40 (0.62÷0.80)	3.11÷5.10 (1.30÷4.75)	3.53 (4.83%)	5.01 (2.19%)	1.63÷5.51 (1.21÷5.43%)	10.20÷10.43 (0.73÷2.97%)
Glucose (RSD%)	50.34÷60.12 (0.74÷3.14)	40.38÷54.26 (0.24÷5.44%)	72.42 (1.07%)	71.35 (1.91%)	40.30 ÷80.82 (0.36÷2.23)	70.32÷131.33 (1.23÷5.96%)
Fructose (RSD%)	31.35÷50.01 (0.57÷1.90)	32.42÷42.34 (1.07÷4.50)	45.00 (1.44)	71.24 (2.10)	60.20÷130.97 (0.97÷6.32)	40.04÷131.12 (1.90÷3.33)
Sucrose (RSD%)	51.24÷152.12 (1.02÷4.84)	70.43÷162.34 (1.51÷2.35)	133.21 (3.62)	111.32 (5.05)	60.60÷314.68 (0.64÷3.56)	73.50÷293.57 (0.39÷4.19)
Maltose (RSD%)	1254.10÷1721.20 (1.49÷4.85%)	2103.10÷3921.40 (0.06-0,59)	2681.34 (0.58)	3922.40 (1.64)	1304.20÷1464.27 (0.11÷2.68)	1660.20÷2331.32 (0,21÷2.48)

Maltulose (RSD%)	8.32÷9.01 (0.36÷4.18)	1.64÷3.83 (0.77÷1.95)	0.95 (4.42)	0.76 (1.02)	9.93÷19.24 (0.02÷2.01)	3.36÷9.45 (0.31÷3.45)
Raffinose (RSD%)	50.01÷60.23 (0.20÷1.90)	60.35÷70.12 (0.35÷1.14)	70.11 (1.78)	72.32 (2.15)	30.21÷141.30 (0.34÷4.28)	30.39÷151.23 (0.13÷4.20)
Stachyose (RSD%)	28.23÷30.27 (0.10-1.14%)	23.23÷40.27 (0.08÷40.27)	30.23 (3.31)	30.42 (0.62)	20.02÷30.47 (0.40÷3.80)	20.93÷30.42 (0.32÷1.22)
Verbascose (RSD%)	0.34÷4.84 (0.01÷2.37)	0.23÷4.22 (0.06÷0.35)	11.32 (4.50)	10.51 (0.79)	2.98÷10.83 (1.21÷3.24)	0.20÷11.12 (0.23÷4.34)

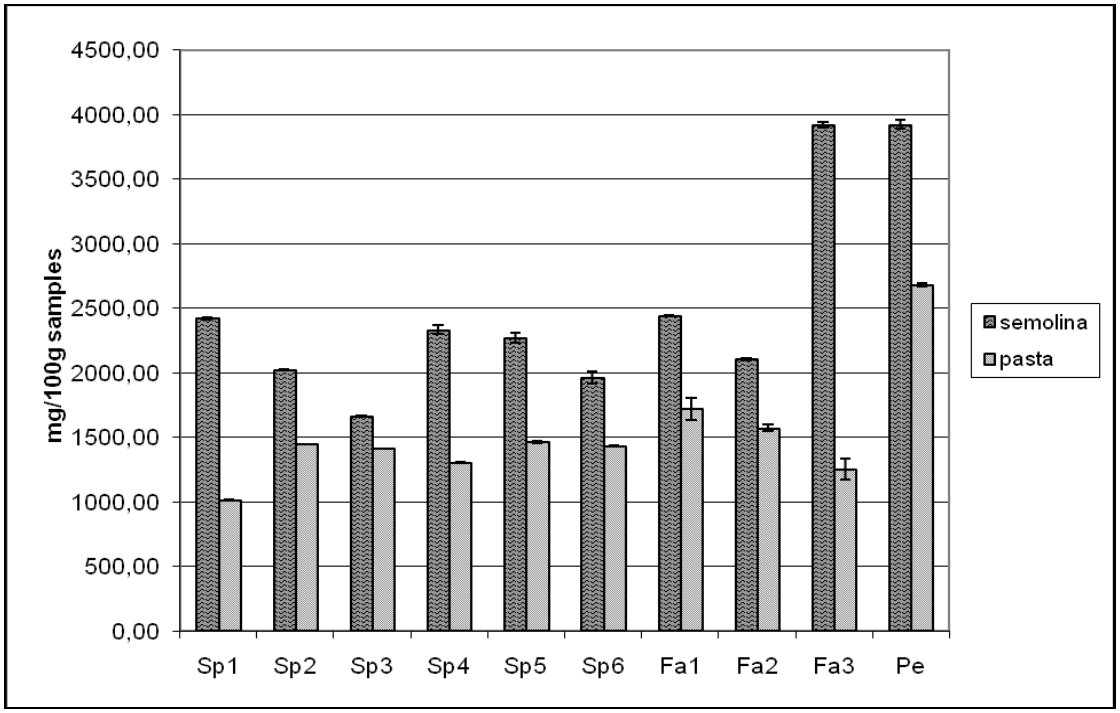

Figure 6. Maltose (mg/100g sample) in pasta and relative semolina.

Table 5-Values of carbohydrates in commercial samples.

	***Spaghetti Mean value (mg/100g)* ±Sd**			***Penne Mean value (mg/100g)* ±Sd**		
	A	***B***	***C***	***A***	***B***	***C***
Galactose	4.33±0.34	4.22±0.48	3.40±0.01	2.30±0.02	6.70±0.01	1.50±0.03
Glucose	112.34±0.32	110.20±0.42	90.32±0.04	70.31±0.32	71.20±0.21	52.30±1.31
Fructose	80.33±0.04	84.30±0.34	60.31±0.42	30.45±0.67	23.40±0.43	13.20±0.35
Sucrose	248.30±0.21	242.60±5.72	140.21±0.14	134,22±2.13	132.32±3.36	93.21±4.63
Maltose	2519.30±40.02	2502.00±34.20	2182,00±30.32	3020.41±52.78	2280.42±55.61	2483.21±43.24
Maltulose	6.23± 0.23	5.98 ±0.15	5.36± 0.11	12.18±0.32	13.12±0.37	14.68±0.42
Raffinose	60.32±1.00	62.30±0.31	32,44±0.14	31.11±0.43	41.22±0.35	12.30±0.04
Stachyose	31.48±0.31	30.28±0.23	20.33±0.15	20.31±0,32	32.49±0,79	12,13±0,44
Verbascose	2.63±0.02	2.03±0.03	1.42±0.04	2.40±0.09	1.20±0.05	1.23±0.04

4.3.4 Principal Component Analysis

Acquistucci, 1996, performed a PCA analysis on lysine, leucine, hystidine, glycine, available lysine, free reducing sugars (calculated after reaction with ferricyanidine and expressed as mmoles maltose per kg samples), furosine, red, yellow and brown indices and observed that sugars contributed few to discrimination while furosine and red index were determinant.

In this work, PCA was carried out on a 16 x 3 data matrix, where 16 was the number of pasta samples and 3 was the number of the variables, that are respectively maltulose, furosine index and PCI index. PCA was performed of the autoscaled date.

Computation of the PCs resulted in the first and second principal components describing 48.7 and 32.8 % of the variability in the original observations, respectively, while both principal components account

for 81.5 % of the total variance. The results regarding eigenvalues and the factor loading are respectively reported in Table 6-A and Table 6-B. While furosine and PCI variables have an high statistical weight on component one with similar negative factor loadings values, maltulose contributes with a value near to 1 to describe the second component. Therefore we could affirm that all the three variables are reliable descriptor of the system.

Table 6-A Eigenvalue results

Number	*Eigenvalue*	*Variance*	*Percentage*
1	1.46009	48.670	48.670
2	0.985049	32.835	81.505

Table 6-B Factor loadings results.

Factor loadings	*Component*	*Component*	*Component*
	1	2	3
maltulose	0.218632	0.966525	-0.13427
furosine	-0.700043	0.0594938	-0.711618
PCI	-0.679809	0.249578	0.689616

T

The bidimensional plot of the sample scores in the space defined by the two first principal components shows a natural separation between pasta of different formats: spaghetti, penne and farfalle samples.

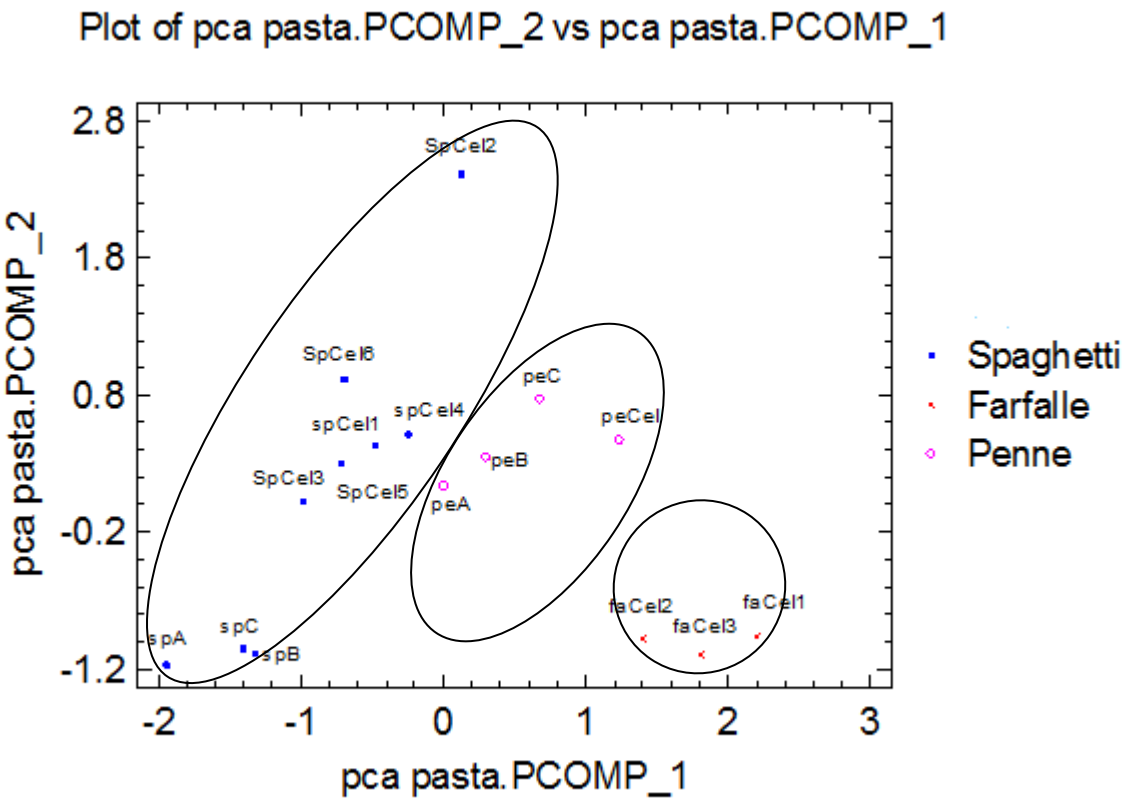

Figure 7. PCA score plot of the 16 pasta samples analyzed. With Sp =spaghetti format; Fa=farfalle format; Pe= penne format. Cel= Cellino samples. A; B, C = commercial samples of three different trademarks.

4.4 Conclusions

A new method was optimized for carbohydrates determinations in pasta samples by HPAEC-PAD.

The high sensitivity and reliability of this techniques had permitted to evidence the presence of maltulose, deriving from maltose isomerization process, not only in pasta dried samples as previously described by García-Baños et al, 2004, but also in fresh pasta samples and in semolina.

Therefore it is possible to think that the only maltulose is not as a good marker of pasta quality. Perhaps, considering the relative differences between pasta and relative semolina maltulose concentration could give a possible indication about drying pasta step conditions, although many notices about semolina quality and storage would be necessary. Furthermore also the only furosine index or colour indices are not sufficient to determine pasta quality, but in association with maltulose determination they could proposed as markers of food quality. In confirm of this, by Principal Component Analysis, it was performed a discrimination between three pasta formats that showed different cook effect values strictly related to drying cycle conditions .

4.5 Acknowledgements

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5. COMPARISON OF DIFFERENT COMMERCIAL FRUCTANS BY MALDI-TOF-MS AND PAD METHODS

5.1 Introduction

5.1.1 Commercial inulin and oligofructose

Many fructans are commercially available with a different name corresponding to various brands: for example native inulin is named Frutafit IQ ™ by Sensus or Raftiline ST ™ by Orafti or Fibruline™ by Cosucra. Inulins are generally extracted from chicory roots of Chicorium intybus, other sources of inuin are Jerusalem artichoke (16-20%) and followed by garlics (9-11%), onions (2-6%), leeks (3-10%) and wheats (1-6%). After extraction in hot waters, all inulins are purified and then spray-dried to obtain final product in powder. In some type of inulin, named high performance inulins, simple sugars or short-chain are removed, as in inulin Frutafit TEX™ of Sensus (Figure 1-2) .Oligosaccharides are obtained from inulins by an endoinulinase hydrolysis and they are classified according to oligosaccharides percentage and simple sugars percentage. Like for inulins, simple sugars could be removed or added. A commercial oligosaccharides mixture named Actilight™ is not produced from inulin but it is is industrially produced through fructosyl-transfer from sucrose using a fungal enzyme.

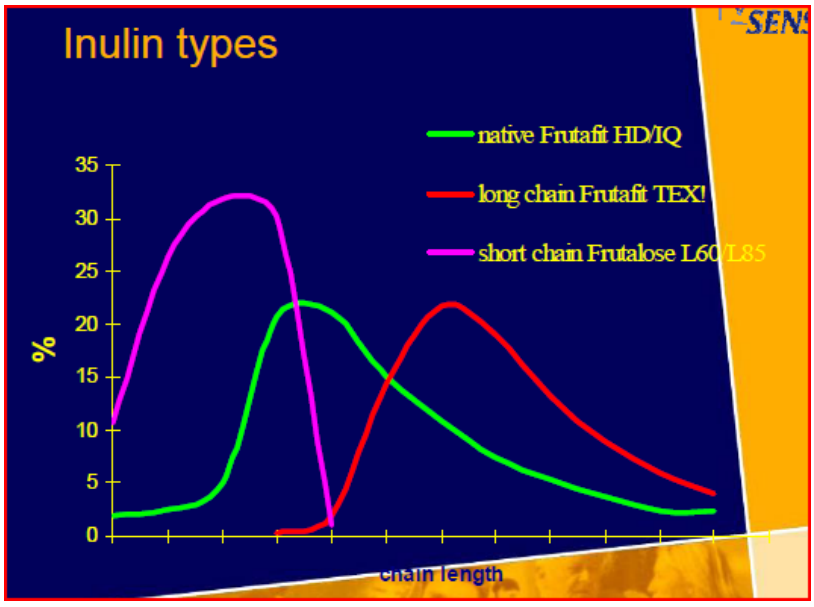

Figure 1. Commercial inulin samples obtained by Sensus

Chicory roots (15-17%inulin)

Washing, Slicing and grinding

-Extraction;
-Diffusion in hot water (70-80°C during 1 hour);

Chicory pulp, salts.wast

Animal feeds

Endoinulinase hydrolysis+storage

Sugars addition

Sugars and short- chain carbohydrates removal

Purification by sterile filtration

Spray-drying

L60* **L85*** **L95*** **P95***

RAFTILOSE family

HP **LS** **GR,ST**

HP-Gel

St-Gel

Raftilose family: oligosaccharides. **(*** The number indicates the oligofructose content; the difference is the sum of glucose, fructose and sucrose) L=liquid; P=powder**.**
There is also Synergy1 that it is oligofructose-enriched Inulin
Raftiline family: inulins HP=High performance Inulin ; GR=Granulated Inulin; ST=Standard Inulin; LS:Low sugar.

Figure 2. Industrial process to obtain inulin and oligosaccharides

5.1.2 Matrix-assisted laser desorption/ionization mass spectrometry for carbohydrates analyses

MALDI is a method that allows vaporization and ionization of non-volatile biological samples from a solid state phase directly into the gas phase.

A pulsed ultraviolet laser beam, most commonly a nitrogen laser with a 337 nm wavelength, serves as the desorption and ionization source in MALDI. The sample is dissolved in a matrix. Matrices are small organic compounds that are co-crystallized with the analyte. The matrix plays a key role in this technique by absorbing the laser light energy and causing part of the illuminated substrate to vaporize. A rapidly expanding matrix plume carries some of the analyte into the vacuum with it and aids the sample ionization process. (Figure 3)

TOF mass spectrometers operate on the principle that when a temporally and spatially well defined group of ions of differing mass/charge (m/z) ratio is subjected to the same applied electric and allowed to drift in a region of constant electric field, these ions will traverse this region in a time which depends upon their m/z ratios. (Steiner and Schaller). Therefore in linear mode the ions travel down a linear flight path (Fig 4-A) .

In the reflectron mode depicted in Fig 4-B, the reflectron or ion mirror located at the end of the flight tube has the function to compensate for the differences in initial velocity of ions with identical m/z values by forcing ions through a curved arc by the use of an electric field, or reflector. Ions with a greater initial velocity penetrate further into the field and therefore have a longer path to the detector than the same ions with lower initial velocities (Sporn and Wang, 1998).

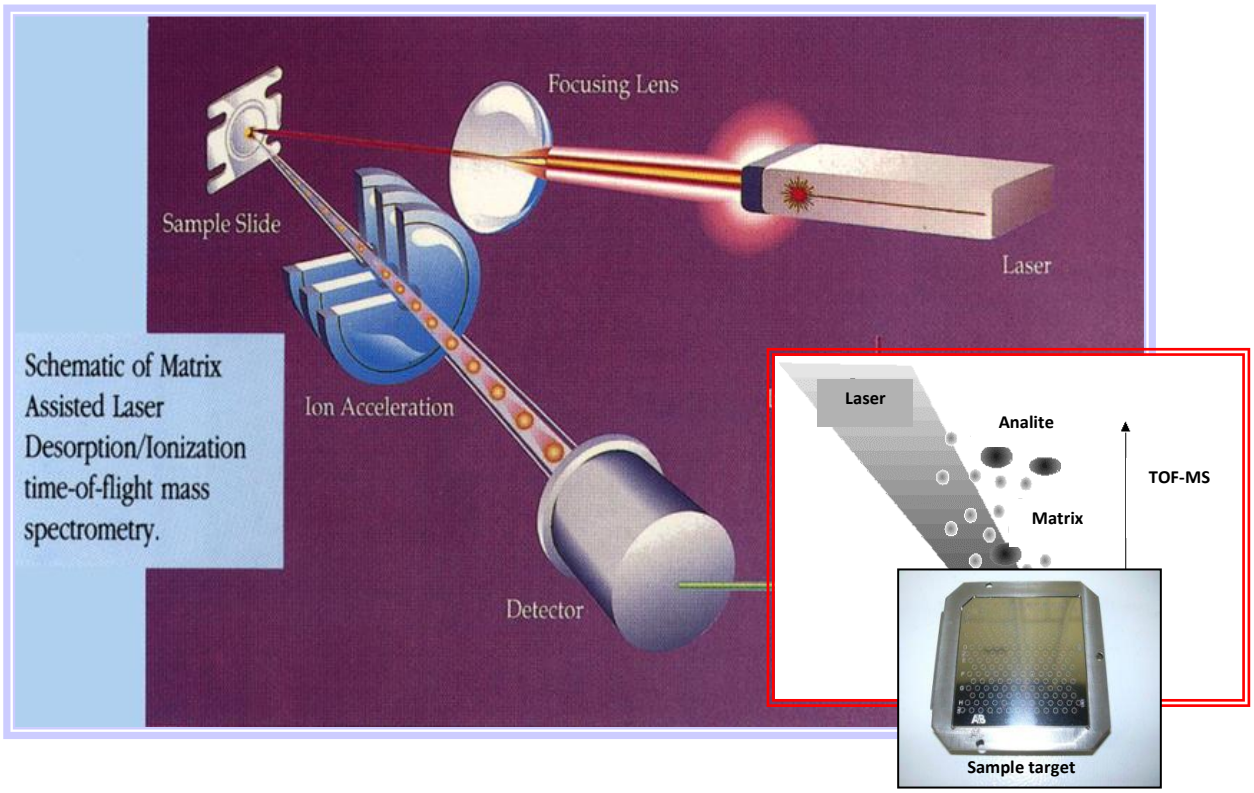

Figure 3-Schematic of MALDI-TOF-MS

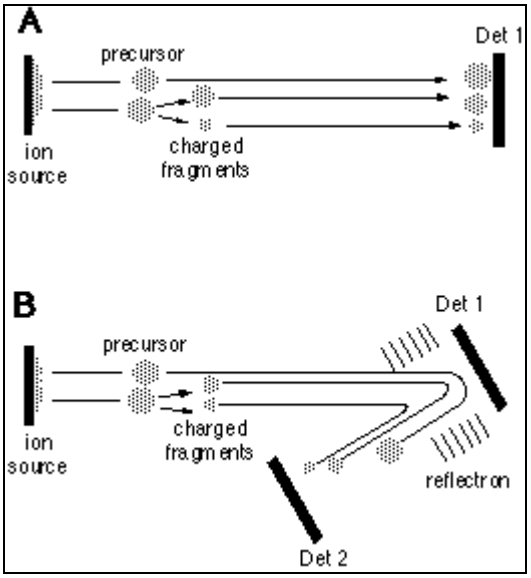

Figure 4 Schematic of the Linear (A) and Reflectron mode (B).

5.1.3 Aims of this work

In this part of work a preliminary screening of different commercials fructans was investigated with the major aim to study oligosaccharides and polysaccharides distribution and the degree of polymerization and to correlate it with, for examples, prebiotic properties or capacity to form gels.

These analyses are conducted simultaneously by HPAEC-PAD and MALDI-TOF-MS to obtain more notices possible about fructans and to compare the two analytical techniques.

Regarding HPAEC-PAD analysis, a great attention was focused on the choice of the column, the mobile phases and on the optimization of the gradient elution to research a good compromise between short time analysis and an high DP detected.

About MALDI-TOF-MS analysis, the choice of the matrix and the samples techniques preparations was optimized in relationship to fructans typology. MALDI-TOF-MS are compared with others mass spectrometric techniques such as Quadrupole Time-of-Flight (Q-TOF) LC/MS and Triple Quadrupole mass spectrometer with electrospray sources.

Finally vegetables (e.g. onions and shallots), that are a natural source of fructans, are studied by MALDI-TOF-MS and HPAEC-PAD.

5.2 Materials and Method

5.2.1 Sample of fructans analyzed

Samples of fructans investigated are:

- Actilight 950P (Beghin Mijie, Thumeries, France);
- Raftiline ST (Orafti, Tienen, Belgium);
- Raftilose P95 (Orafti, Tienen, Belgium);
- Frutafit IQ(Sensus, Roosedaal, The Netherlands)
- Frutafit TEX (Sensus, Roosedaal, The Netherlands).

5.2.2 MALDI-TOF-MS analyses

MALDI-MS measurements were performed using an MALDI-LR time-offlight mass spectrometer (Micromass, Manchester, UK) operating both in the positive linear ion mode and in reflectron mode.
Ions formed by a pulsed UV laser beam (λ = 337 nm) were accelerated at 15 keV.
The choice of laser strength is very important because it determines the degree of the desorption and ionization of the analytes. Generally laser strength has been chosen based on the signal-to-noise ratios: low laser power may lead to insufficient signal-to-noise ratio while an high laser strength can lead to more fragmentation and when it increased over a certain amount, the resolution deteriorated rapidly (Wang, Sporn, 1999). Therefore laser strength was varied from sample to sample to obtain the best signal.
The properties that it could have the matrix it is high absorbivity, high concentration (optimal molar ratios are in the 1:1000 to 1:10000 range) , solubility in the same solvent of analytes, inertia toward analyte.
The choice of the matrix and the samples preparation techniques are very critical steps in MALDI-MS analysis.
For all samples, different matrices were tested: 2,5-dihydroxy benzoic acid (2,5-DHB) (Sigma-Aldrich), trihydroxyacetophenone (THAP) and 3-aminoquinoline (3-AQ), that they are recommended as matrixes for carbohydrates analysis.
Matrices are prepared at different concentrations (10mg/mL; 20mg/ml; 100mg/ml or saturated) in various solution: HPLC-grade water; water/acetonitrile (50/50 w/w) mixture; water/ethanol (90/10 w/w) for the dried droplet techniques and in acetone for fast evaporation technique. Trifluoroacetic acid (TFA) was added to all samples solutions in a percentage of 0.1% to promote the ionization.
Different fructans concentration are prepared (0.1 mg/mL; 0.5 mg/mL; 1 mg/mL; 4 mg/mL; 10 mg/mL) in HPLC-grade water and filtered on a 0.45 μm membrane filter.
A solution of the alkali ion salt sodium chloride (0.01M) was added to some samples to obtain a single molecular ion peak. Three replicated measurements were performed on each sample.

With the aim to obtain a correct calibration in the wide range of mass molecular interest, an external calibration was performed using the $[M + H]^+$ ions of a peptide mixture (angiotensin I, angiotensin II, substance P, rennin, ACTH, insulin bovine, cytochrome c) (Sigma-Aldrich) .

Both dried droplet and fast evaporation sample preparation techniques are investigated, with the aim to verify which gives the best results in terms of a good quality spectra and in relationship with the type of fructans.

5.2.3. HPAEC-PAD analyses

For analysis of both oligo and polysaccharides the column choose was a Carbopac PA200 (Dionex). The mobile phase consisted of deionized water (eluent A), 600 mM aqueous sodium hydroxide (eluent B), and 250 mM aqueous sodium nitrate solution (eluent C), employing a gradient program as reported in Table 1.

Table 1. Gradient elution optimized for fructans analysis

Elution time (min)	A(%)	B(%)	C(%)
-40 [a]	89	10	1
0	89	10	1
4	84	15	1
25	79	15	6
80	79	15	40
80.1	0	50	50
90	0	50	50

[a] Negative time indicates time prior to sample injection.

5.3. Results and discussion

5.3.1 MALDI-TOF Analyses

MALDI-MS was originally developed for measuring the mass of large molecules such as proteins, but since about 1991 it has also been applied to carbohydrates (Wang and Sporns, 1999).

The first type of fructans analyzed was Actilight 950P™. Actilight™ is defined by the producer Begin Meiji as a mixture of fructooligosaccharides with GF chains between 2-4 that it is industrially produced through fructosyl-transfer from sucrose using a fungal enzyme. Furthermore the following composition of dry substance was reported: 0.3% fructose, 0.4% glucose, 3.0% sucrose, 36% 1-kestose (GF2), 49% nystose (GF3), and 12% fructosylnystose (GF4) (Krol and Grzelak, 2006). This FOS mixture was also chose to test the reliability of MALDI-TOF results. MALDI-TOF mass spectrum, as reported in Figure 5, showed a maximum degree of polymerization of 6, giving an unit of fructose mass (162 Da) higher than it was labelled.

As reported in literature (Wang and Sporns, 1999) the sample, with no modification to the matrix, contain both sodium and potassium ions resulting in multiple carbohydrate peaks. The peak intensity of sodium adduct $[M+Na]^+$ was higher than for all oligosaccharides with DP range between 3-6.

The peak intensity of carbohydrates alkali-metal ion adducts is dependent on the concentration of the alkali-metal ions in final solution applied to the probe and on the affinity between the metal and the carbohydrate. It has been shown that the affinity of alkali metals to carbohydrates follows the order H<Li<Na<K<Cs. Sodium is a ubiquitous contamination of matrix solutions and sample targets.

The selection of matrix that it is a very critical step in MALDI- MS analysis was based on a comparison of spot-to-spot or sample-to sample repeatability and ability to reach a good quality spectrum with reasonable signal-to-noise ratio and the best resolution. Dried droplet was selected as sample preparation technique because it is simple and rapid and it does not require special tool.

Different matrices are investigated, like DHB and 3-aminoquinoline.

With the use of DHB a good quality spectra was obtained, as elicited in Raftilose™ spectrum (Figure 6), with a maximum degree of polymerization of 10, confirming that this soluble fiber is an oligosaccharide as reported in label and giving further information about DP and FOS distribution.

With DHB the repeatability was acceptable, although Mohr et al, 1995 noted that DHB crystals formed only in near the rime of the probe, complicating the location of suitable laser ionization positions because in the center of the probe only a few crystals could be found.

Potassium adduct was not found after DP 7 and generally sodium adduct was predominant.3-AQ matrix gave a best result in terms of maximum DP showed (Figure 7) :12 instead of 10.

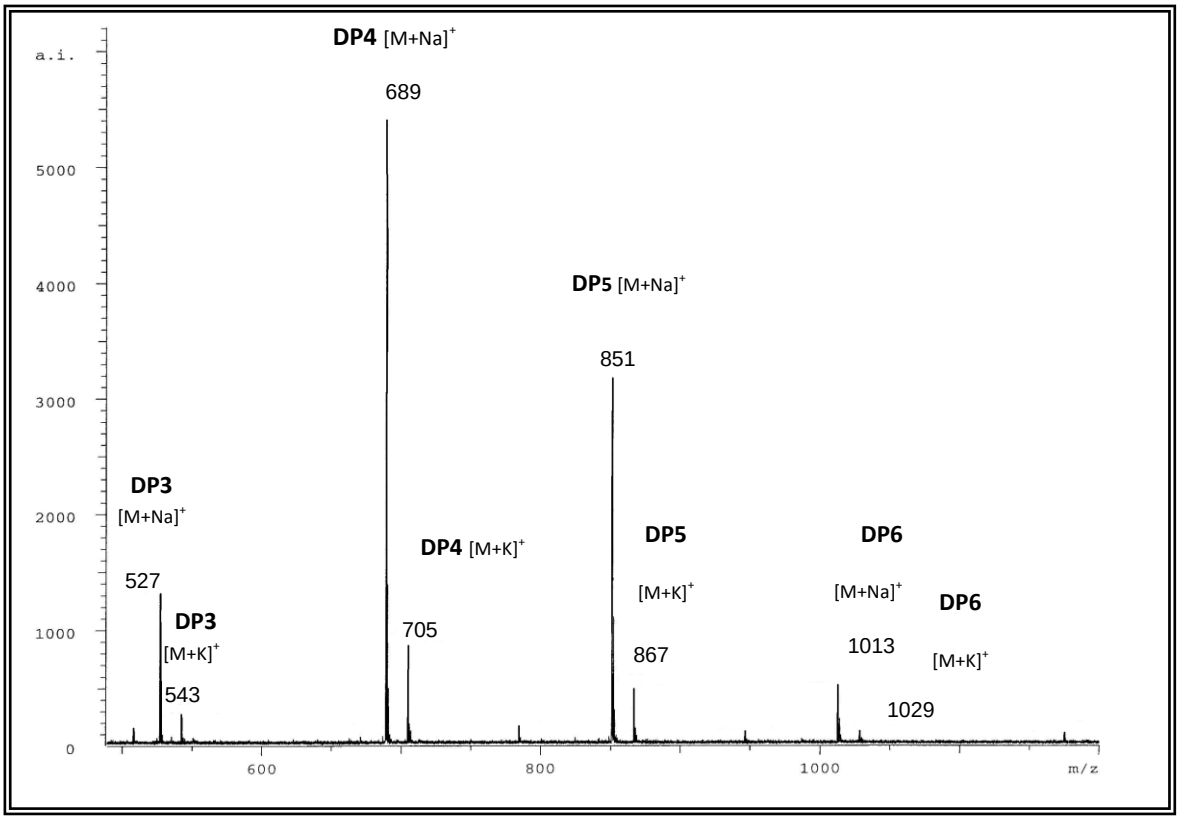

Figure 5 . MALDI-MS spectrum of a standard solution of Actilight (Matrix:*: 2,5-dihydroxy benzoic acid (DHB). Sample was prepared by Dried Droplet technique*

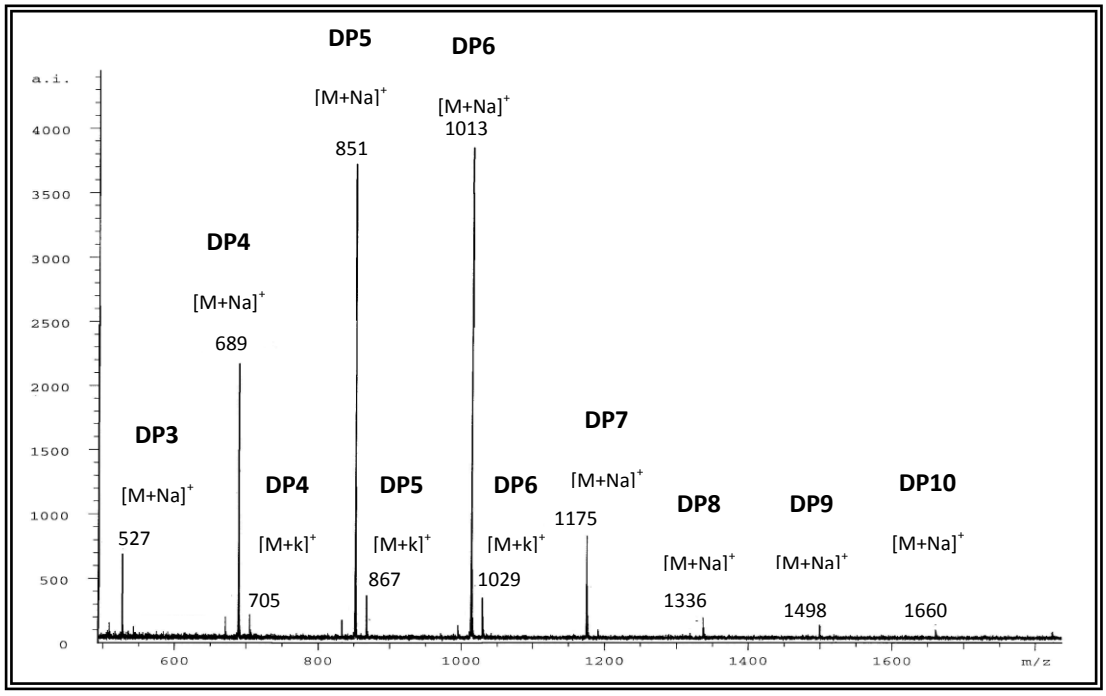

Figure 6. Raftilose™ spectrum (1mg/mL). Matrix DHB (water/acetonitrile (50/50v/v) trifluoroaceticacid (TFA) (0.1% v/v) mixture. Dried droplet sample preparation technique. Linear mode.

Stahl et al, 1997 showed that 3-AQ is more sensitive to contaminants such as salts. We found that in the first part of spectrum, until mass of 600 Da , it is difficult to attribute the signals to analyte or to matrix and therefore to attribute degree of polymerization.

Another type of fructans we have studied was Raftiline ™ , that it is declared from the producer as a native inulin obtained directly from chicory roots after purification: the spectrum reported in Figure 8 showed that its maximum degree of polymerization is 27 (corresponding to m/z=4418). An higher DP it was not found because an high background decreased the signal/noise ratio.

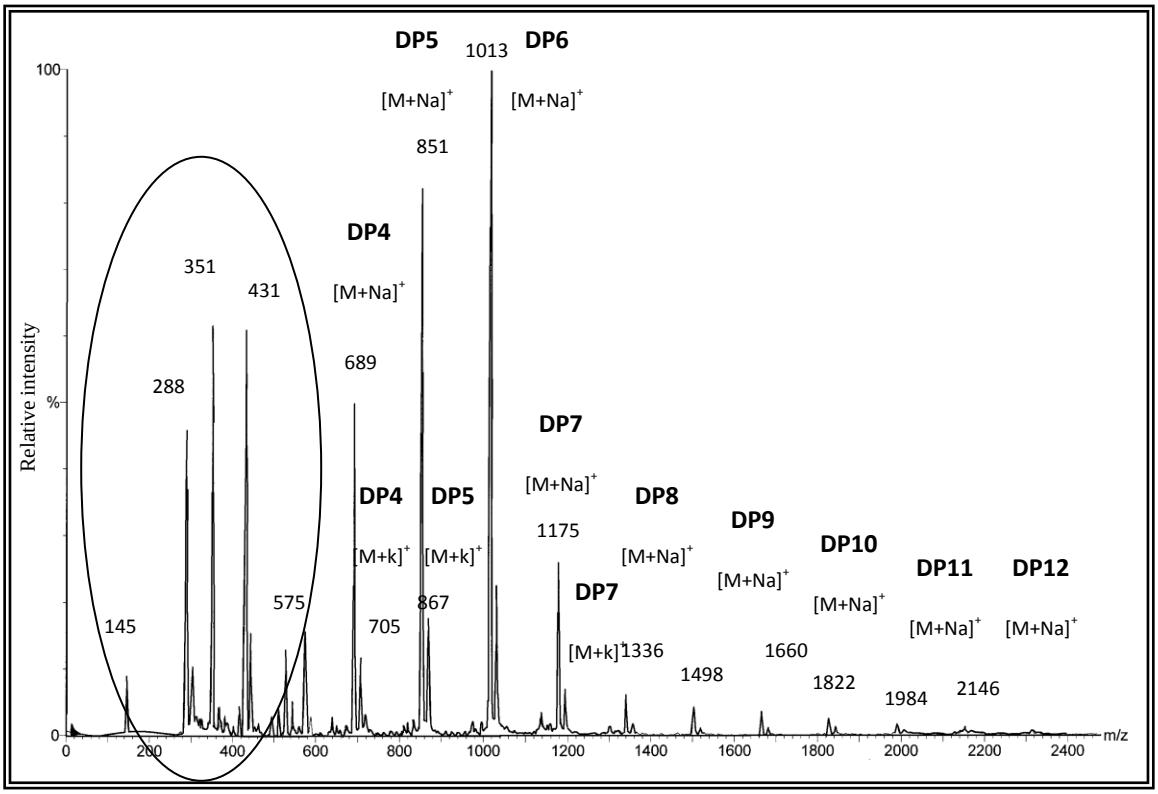

Figure 7. Raftilose™ spectrum (1mg/mL). Matrix 3-AQ (water/acetonitrile (50/50v/v) and TFA (0.1% v/v) mixture. Dried droplet sample preparation technique. Linear mode.

In this inulin oligosaccharides fraction is more preponderant than polysaccharides fraction (that it was zoomed out of 15) with a maximum relative intensity in the range between DP 3-6.

This spectrum was obtained with 3-AQ matrix adding sodium chloride. In fact to obtain a single alkali ion adduct peak, it is possible to dissolve carbohydrates in a 0.01M solution of the alkali ion salts. Concentration of alkali ions it is crucial since too high a concentration of salts could also suppress the molecular ions. The formation of a single adduct it is very important if the aim is a quantitative analysis; furthermore it simplify the spectra interpretation also in qualitative analysis.

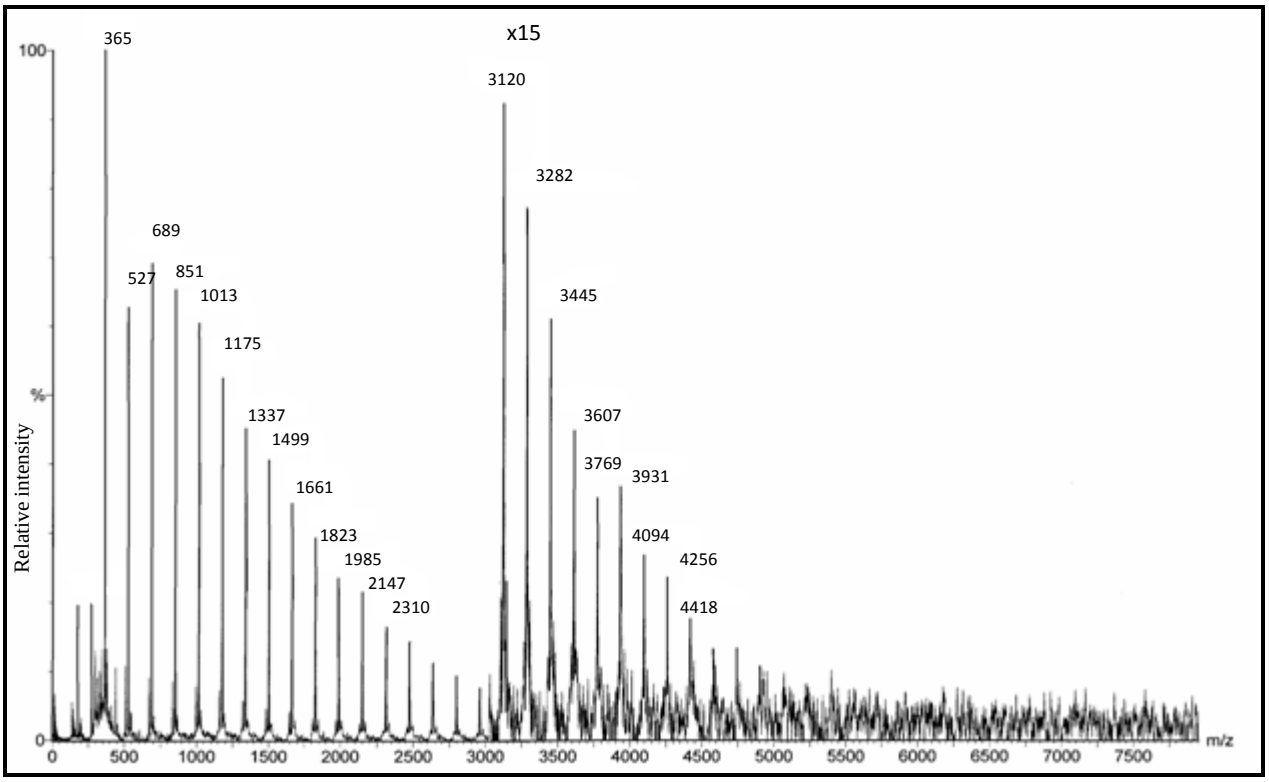

Figure 8. Raftiline™ spectrum (1mg/mL; NaCl 0.5mg/mL).Matrix 3-AQ (water/acetonitrile (50/50v/v) trifluoroacetic acid (TFA) (0.1% v/v) mixture. Dried droplet sample preparation technique. Linear mode.

Another type of inulin, named Frutafit TEX™ is analyzed by MALDI-TOF and it has a maximum DP about 44 corresponding to m/z 7170 (Figure 9). We could observe not only the differences in the DP number between Raftiline™ and Frutafit TEX ™ but also about oligo and polysaccharides distribution.

The maximum relative intensity of Frutafit TEX ™ is in the ranges between DP 12–14 and polysaccharides fraction has a more intensity respect than oligosaccharides fraction. This is probably to attribute to the fact that Frutafit TEX ™ is obtained by removing partially simple sugars and short chains. For this type of inulin the best matrix was THAP. In literature it was reported the high solubility of THAP in acetone and the fast evaporation of the latter giving fine crystals and homogeneous incorporation of sample. This techniques resulted in high quality MALDI-MS spectra with high spot-to-spot repeatability and it could be used to resolve the oligosaccharides in inulin up to a mass of 9000 (Careri et al, 2002) .MALDI-MS-TOF could be applied also for fructans analysis in

vegetables where are naturally present, for example onions and shallot, which an example of spectrum is reported in Figure 10.

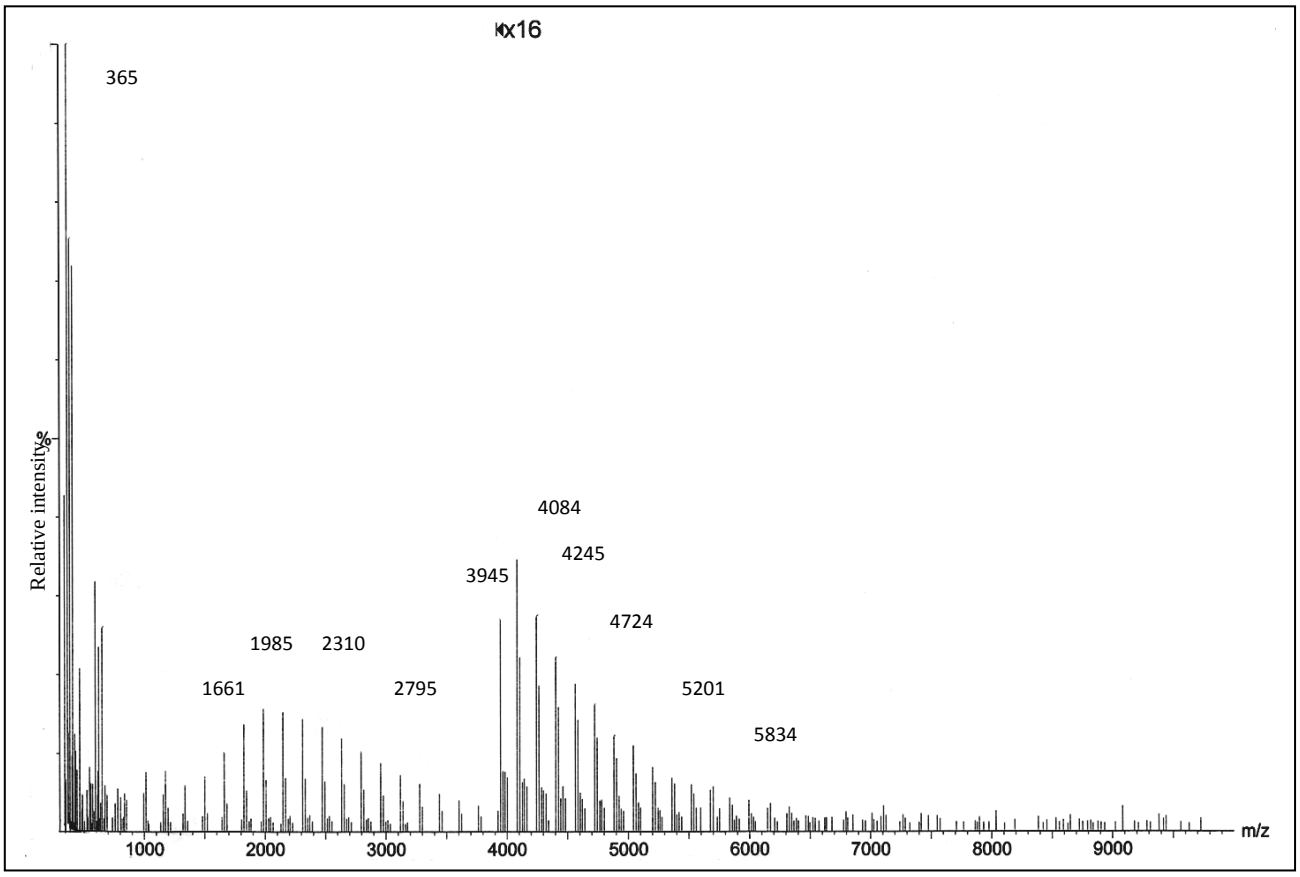

Figure 9. Frutafit TEC™ spectrum (4 mg/mL in deionized water).Matrix THAP (acetone and TFA (0.1% v/v) mixture. Fast evaporation sample preparation technique. Linear mode.

Considering that onion and shallot bulbs contain a high concentration of potassium ions (1.3 mg/g fresh weight), fructans were recovered mainly as monopotassium adduct ions. The maximum degree of polymerization obtained, with 3-AQ matrix, was 17 for shallots, 12 for Tropeana red onion.

For this analysis reflectron mode gave better results than linear mode: it is known that the reflectron can improve resolution up to 6000 in comparison to linear mode. However the principal problem

associated to reflectron is that ions that fragmented in the long drift tube were lost, reducing the signal (Sporn and Wang, 1998) .

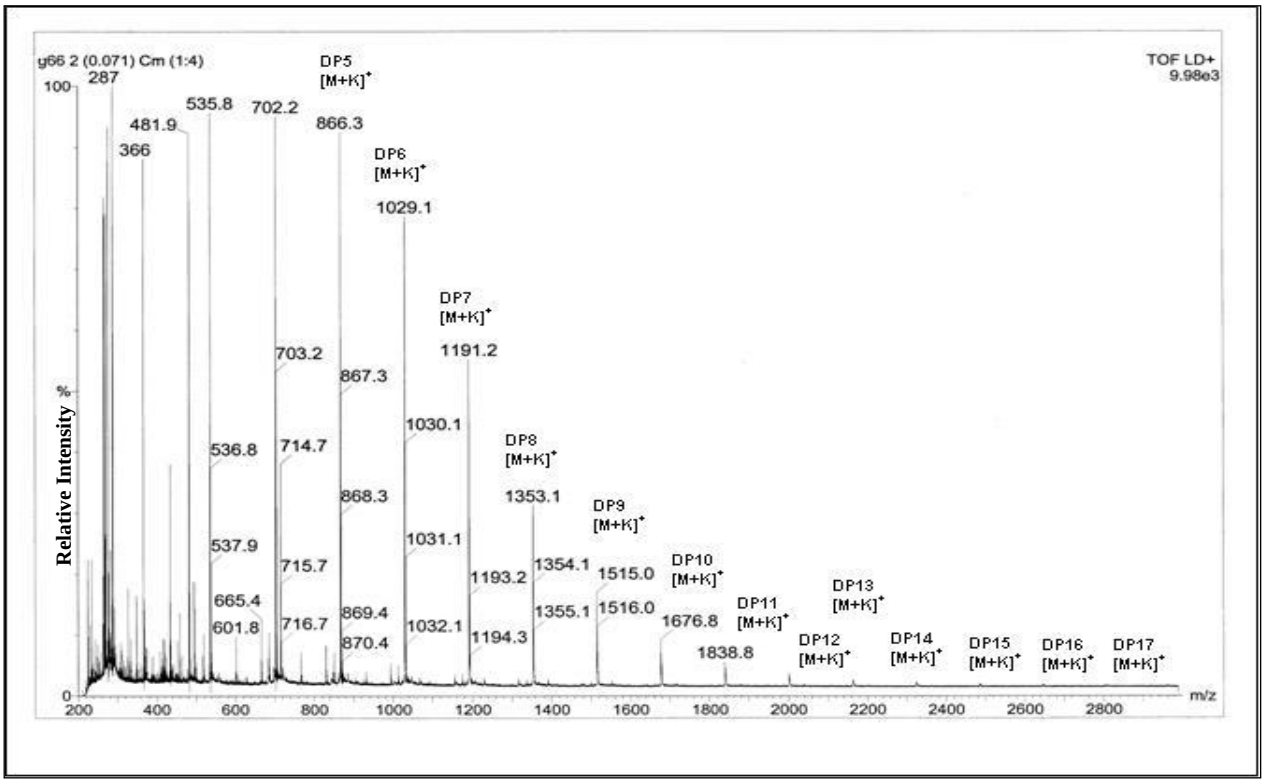

Figure 10. Shallots (diluted 1:3 with deionized water) spectrum obtained in Reflectron Mode. Matrix: 3-AQ.

5.3.2 Comparison between HPAEC-PAD and MALDI-TOF-MS

In HPAEC- PAD analysis it is very important to optimize the choice of the column, the gradient of elution with the aim to develop an accurate and valid method for the quality evaluation of fructans at different DP. The quality of chromatograms was considered evaluating the number and the resolution of peak detected.

The use of the sodium nitrate as pushing agent in substitution of the more employed sodium acetate in HPAEC-PAD analyses as previously reported by Zhang et al, 1997 (Zhang et al, 1997), had permitted to separate oligo and polysaccharides with a very good resolution of the polymers in a shorter analysis time. Employing a Carbopac PA200 column, more specific for polysaccharides analysis than the Carbopac PA1 and Carbopac PA100 columns, both oligosaccharides with a low degree of polymerization and inulins with an higher DP were separated in a total rum time analyses of 80 minutes (Figure 11).

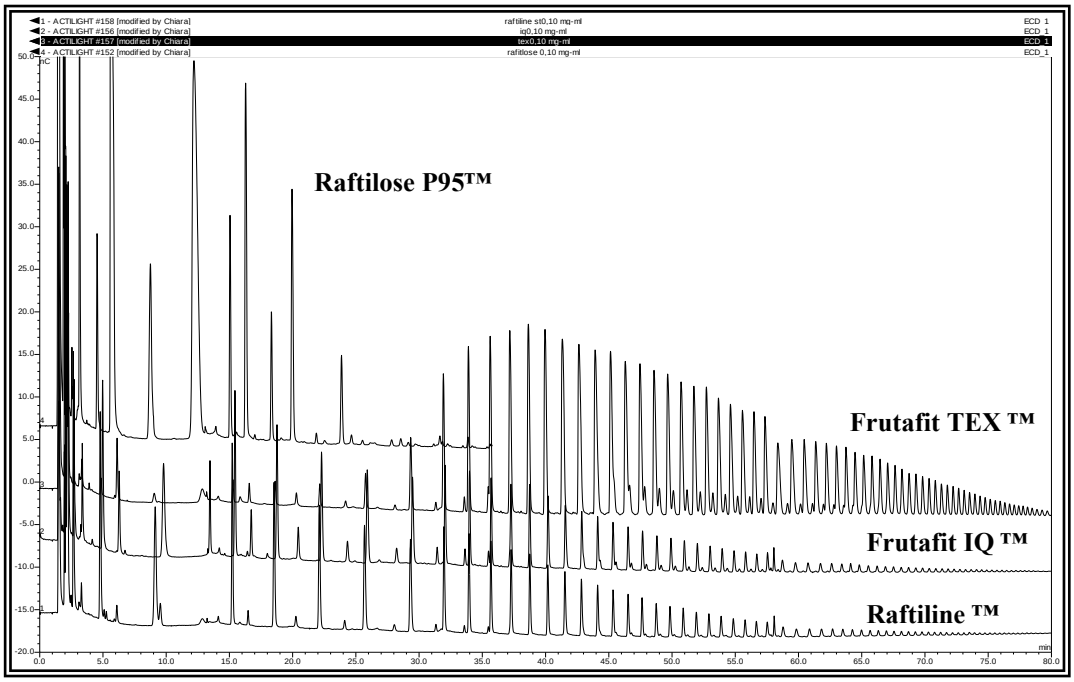

Figure 11. Chromatogram profiles of Raftilose P95™,Frutafit TEX™, Frutafit IQ™ ,Raftiline ™

Instrumental precision was checked from six consecutive injections of Frutafit IQ solution; the relative standard deviations (RSDs) obtained were lesser than 2.7%.

Respect than MALDI-TOF-MS analysis it is not possible to attribute the correct molecular assignment and consequently the degree of polymerization of fructans. In fact not fructooligosaccharides standards are commercially available with a DP>5 and therefore the assignment of the chromatographic peaks with DP higher than 5 was based on the generally accepted assumption that the retention time of a homologous series of carbohydrates increased as the DP increased, and that each successive peak represented a fructan which had a fructose more than that of the previous peak. This is because retention time increases as the number of negatively charged functional groups concurrently increases (Lee, 1996). A comparison between different fructans was possible by fingerprinting chromatographic profiles in order to evaluate oligo and polysaccharides distribution.

As previously observed in MALDI-TOF-MS analysis, Raftilose P95™ is a fructoligosaccharide with DP ranging from 3 (1-kestose) and 10 (1-F-(1-b-Dfructofuranosyl-7-nystose) and it contained 5% of simple sugars glucose, fructose, sucrose.

The other fructans reported in Figure 11 are inulins that are different for maximum degree of polymerization, oligo and polysaccharides ratio and distribution. The variety of chemical and structural conformations that characterize FOS and inulins influences the capacity to develop a gel-like structure with a white creamy appearance, which can be easily incorporated into foods to replace fats and therefore decrease calorie intake (Teeuween et al, 1992, Chiavaro et al, 2007). Furthermore, the fructans DP can also be correlate to some of their intrinsic properties such as digestibility, prebiotic activity, sweetening power (Rossi et al, 2005) .

In chromatographic profiles depicted in Figure 11, there is the presence of small peaks eluting among GFn monomers that could correspond to isomers composed only of fructose unit chains (inulooligosaccharides) (Stikarovska, Chmelik, 2004), as well as slightly branched fructans (Zhang et al, 1997). In Actilight sample (chromatographic conditions are reported in Materials and Methods, chapter 7) it was possible to observe a small percentage of inuloligosaccharides that are eluted after fructooligosaccharides with the same DP (Figure 12). The method of identification of these peaks will be described in the Chapter 6.

Comparing HPAEC-PAD with MALDI-TOF-MS it is possible to affirm that they are two complementary techniques. The first one had permitted to study an higher number of oligomers and to differentiate fructans with various chemical structure, separating, for example, inulooligosaccharides from fructooligosaccharides; the second had given a better assurance of correct DP.

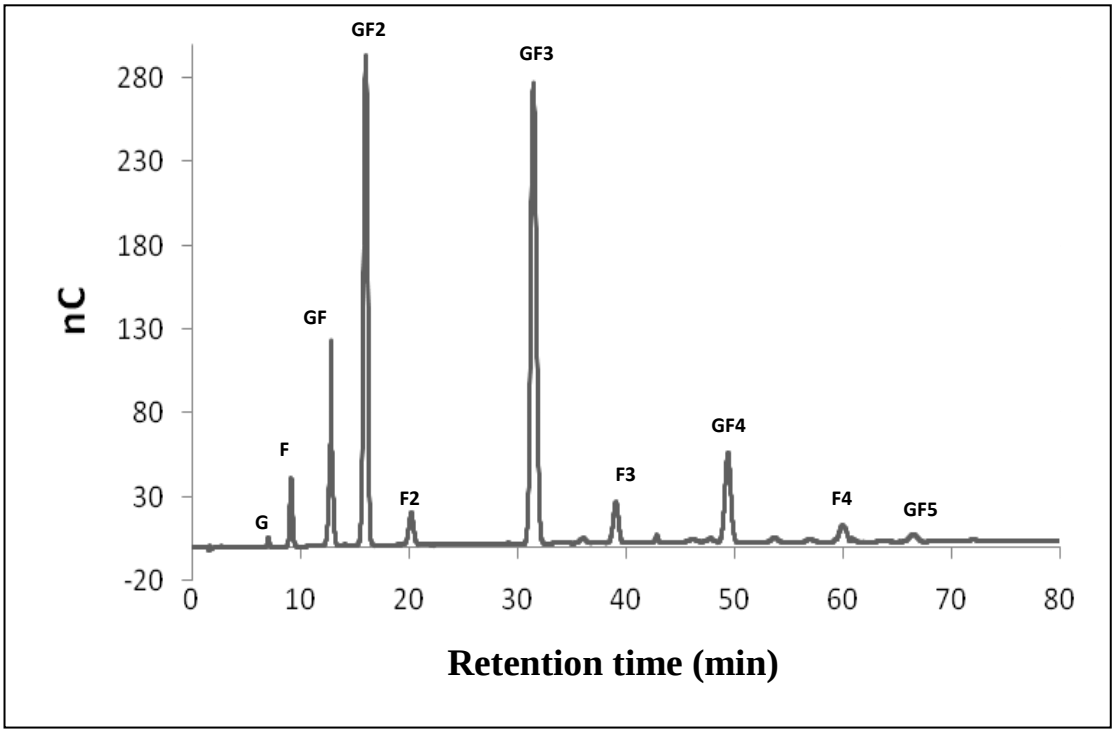

Figure 12. Chromatogram of Actilight™ sample. F= inulooligosaccharides; GF=fructooligosaccharides; with n= number of fructose molecules.

The two analytical techniques showed the same qualitative results regarding the differences of DP and geographical profiles between fructans.

MALDI-TOF-MS is a somewhat faster analysis method than HPAEC-PAD and it is more tolerant to impurities, is largely tolerant to salts and inorganic buffers but it doesn't permit to distinguished masses branched and linear isomers. MALDI is a very useful technique for the analysis of vegetables that are source of fructans of which no notice about chemical structure and degree of polymerization is possible to know beforehand.

HPAEC-PAD was reported to be more sensitive in terms of detection limit than MALDI (Wang and Sporns, 1999), although Onofrejová and Preisler, 2009, reported a very low limit of detection of 0.94 pg analyzing raftilose by MALDI TOF MS.

Regarding quantitative analysis the main problem in MALDI-TOF-MS is poor shot-to-shot repeatability or crystal inhomogeneity. The signal intensity is a very complex function of many parameters, such as sample morphology and laser energy, that strongly influence desorption and ionisation efficiencies. This usually leads to large errors in quantification.

Furthermore ion abundances of carbohydrates varied with the molecular size; in general, the ionization efficiencies of carbohydrates tend to be enhanced by increasing the size (Sung-Seen et al, 2009). Therefore in MALDI-TOF-MS the application of an internal standard is desirable in order to correct for variations in sample preparation and instrument precision.

Onofrejová and Preisler, 2009, reported the quantification of Raftilose™ by the use of the tetraoligosaccharides nystose as internal standard, although is a component of the FOS analyzed, employing an artificial neural work.

In HPAEC-PAD quantitative analysis, the principal problem is that not commercial fructooligosaccharides with DP>5 and inulooligosaccharides are available. Official AOAC method for fructans determination in foods consists of enzymatic hydrolysis using *β-fructofuranosidase* to break down fructans into monosaccharides (glucose and fructose) and subsequent analysis by either spectrophotometry or HPLC (Andesen and Sørensen, 2000, Steegmans at al, 2004, Quemener et al, 1994, Corradini et al, 2004). This method permit only total fructans quantification and not the determination of the single monomers.

In our work we have proposed a method to quantify each oligomers with different DP, as it will described in the Chapter 6, after a preliminary study of pulsed amperometric detector in relationship with degree of polymerization. The response of PAD decrease with DP increasing and also is different between FOS and IOS and between linear FOS and branching FOS with (2→6) link: for example the branched glucose oligosaccharides (e.g. isomaltose) have significantly greater PAD response than the linear mass equivalents (like maltose) (Wang and Sporns, 1999) .

6.3.3 Comparison between MALDI-TOF-MS and other MASS SPECTROMETRY techniques

Comparing MALDI-TOF-MS spectrum (Figure 13) with spectra obtained by Triple Quadrupole mass spectrometer (Figure 14-A) and Quadrupole Time-of-Flight (Q-TOF) LC/MS with electrospray sources (Figure 14-B), it is possible to observe that the best signal to noise was obtained by MALDI-TOF.

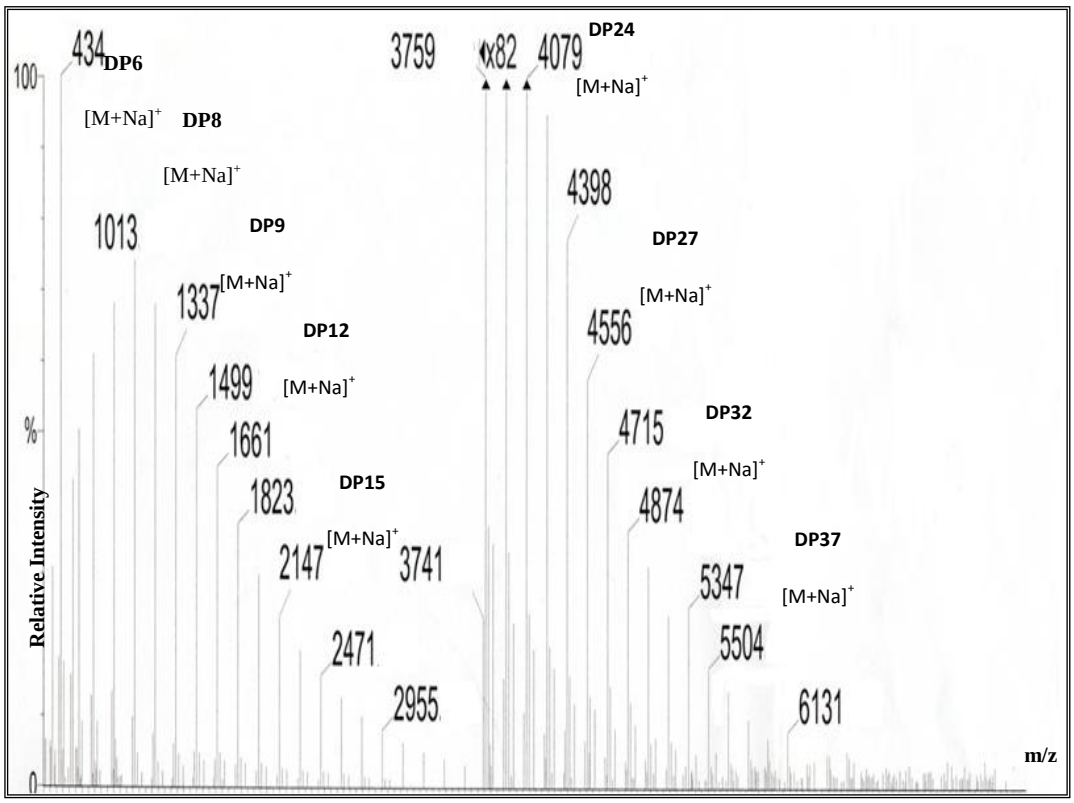

Figure 13: Frutafit IQ ™ (1mg/mL; NaCl 0.5 mg/mL) MALDI mass spetrum *(Matrix:3-AQ)*

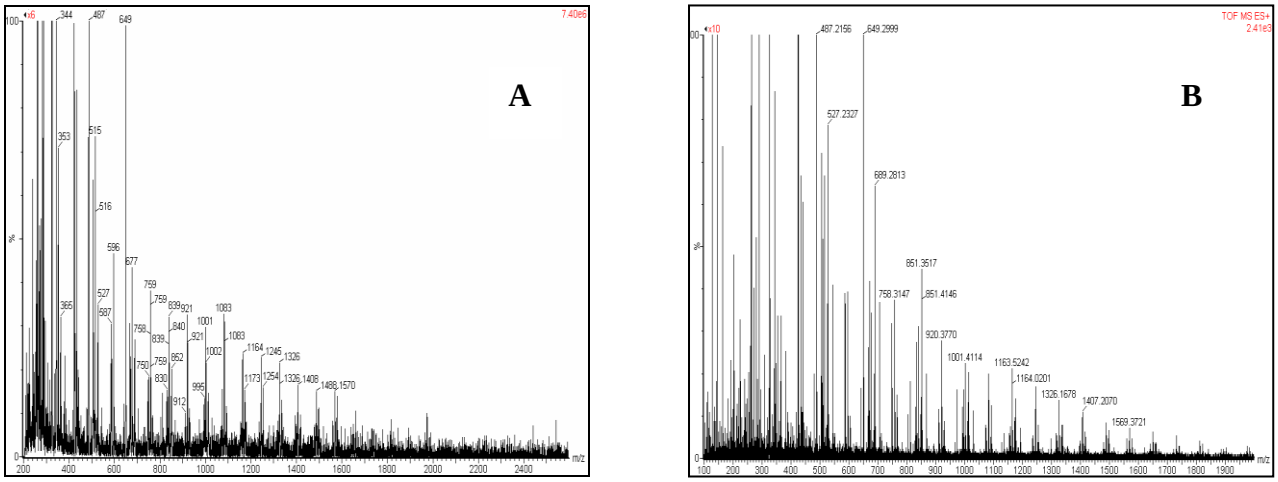

Figure 14: Frutafit IQ™ (1mg/mL) A:Triple Quadrupole mass spectrum B: Q-TOF mass spectrum

MALDI is more tolerant to salts while ESI source is sensible to detergents and inorganic buffers. Unlike electrospray, the domain of which is on-line coupling to liquid-phase column separations, MALDI is routinely used for off-line high throughput (Onofrejová et al, 2009) .

Interfacing anion exchange chromatography with mass spectrometric detection is a very critical and difficult process because typical alkali acetate and hydroxide eluents are not compatible with atmospheric pressure ionisation (API) ,due to their non-volatility and high conductance. For this reason a desalting device occurs between the column and the ESI source and it could contribute to increase the noise. Furthermore neutral carbohydrates are difficult to ionize and, to enhance sensitivity, it occurs to add salts like LiCl (Bruggink et al, 2005) .

5.4. Acknowledgement

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6. STUDY OF PULSED AMPEROMETRY DETECTOR

6.1 Introduction

6.1.1 .State of the Art

The electrochemical behavior of carbohydrates can be affected by molecular weight and structure; this has been demonstrated for various carbohydrate oligomers. Indeed, as stated by LaCourse, 1997, "all quantitative applications of LC–PED must be based on careful calibration plots using standard solutions for each sample component". Unfortunately, for fructans analysis, no commercially standard with a degree of polymerization higher than 5 are commercially available. Quantitation of individual inulin oligomers requires a knowledge of the PAD response factors. In fact, the electrochemical behavior of the detector can be affected by molecular weight and structure: B.Einze and W.Praznik, 1991, demonstrated that the sensitivity of the PAD decreases clearly from DP2 to DP8; Kang et al , 1999, reported that Fn and GFn molecules do not have the same response factor. In order to achieve PAD molar response and to quantify inulin in foods, some methods have been proposed: the most employed involve enzymatic hydrolysis of all fructans to fructose and glucose (Hoebregs, 1997 , Roberfroid et al, 1998).

AOAC Methods (Quemener, 1994: Hoebregs, 1997) involve tree step: an initial water extraction at 85° C for 15 minutes to determine free fructose and glucose, treatment of the residue with amyloglucosidase to convert starch and maltodextrins to glucose and treatment of this residue with inulinase to convert fructan to fructose and glucose. Released sugars are determined at each stage and the concentration of glucose and fructose released from the fructans calculated by difference from the three determination.

These methods of evaluation of the content, however, do not allow for the quantification of the individual oligomers. Another method was reported by Timmermans, 1994, in which the PAD relative response was calculated as function of DP for quantitative analysis of the individual compounds, using sucrose as internal standard and determining the number average DP and weight average DP (DPw) of inulin from chicory .

In this work it is reported that the sensitivity of PAD detector decreases clearly from DP2 to DP8 while, for longer oligomers (DP 11-17), it decreases only slightly.

This method has the disadvantage to need of a preparative RP-18 HPLC chromatography to isolate oligomers.

Recently Abballe et al, 2007, reported the electrochemical response of some dextrans based on the complementary use of the HPAEC-PAD and CZE-UV.

The calculation of the PAD response for each oligosaccharides is necessary before performing fructans quantitative analyses.The determination of the single oligomers is important, for example, for the study of fructans capacity to stimulate the growth of bifidobacteria, because they are strictly related to DP (Rossi, 2005). The degree of polymerization can also influence prebiotic activities, digestibility, caloric value, sweetening power, water binding capacity (Lopez-Molina et al, 2005), formation of gel or fat replacer.

6.1.2 Inulin as functional ingredient added to cooked ham

Cooked ham quality depends on many factors like raw meat, brine composition and it is related to mode and conditions of mechanical and thermal treatment.

In Figure 1 it is reassumed the industrial process for cooked ham production.

Firstly legs of pork are received and stored. The ingredients for preparation of the brine are then received and the brine is prepared. The first step in the production process is the removal of the bones. The bones can be removed by "unchopped ham technique" that permits an high-quality ham without polysphosphates addition.

The meat placed on conveyor belts, is passed beneath the head of a multi-needle injector, which injects brine into the meat. At the end of injection, the meat is passed into the massagers, for a range of 30-70 hours, favoring the distribution of the brine through the entire muscle and to facilitate cohesion of the various muscles and pieces of meat during cooking.

Once it has been worked and softened, the meat leaving the massagers needs to be shaped.

Insert in a specific heat shrinking bag, then a vacuum is created and the bag is clipped and immersed in boiling water for heat shrinking and then appropriately inserted in a cooking mould leaving hanging a part of the bag which will have the task of collecting the cooking juices.

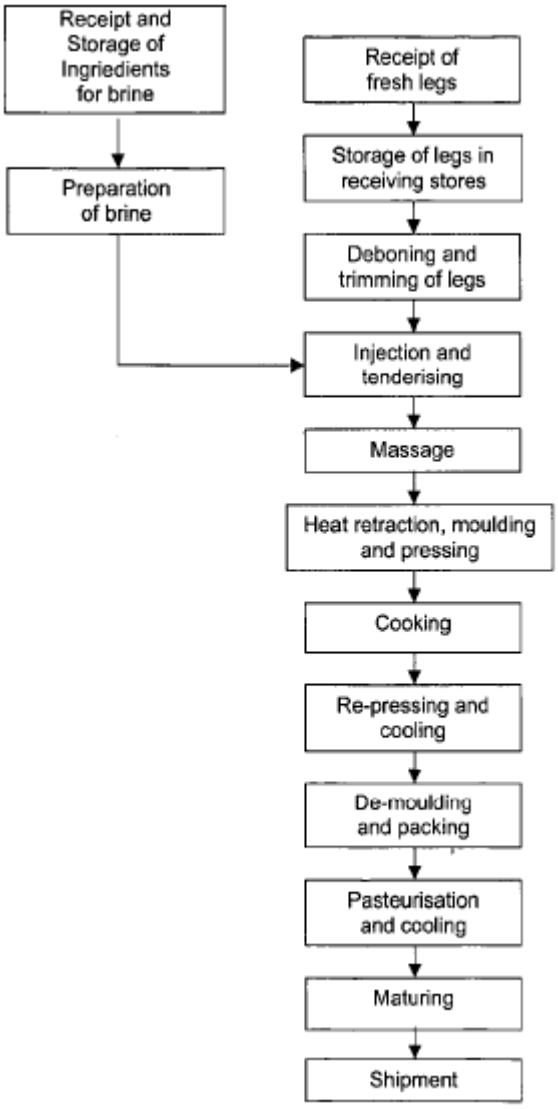


Figure 1. Industrial process for cooked ham production

Cooking takes place in steam ovens, with operating conditions according to the internal temperature of the muscle. The ham are then cooled in a refrigerator for about 24-48 hours.

Removal from the moulds takes place manually. A process of pasteurization was conducted on the surface, placing the hams in autoclaves for 20-30 minutes, then they are cooled. Finally the ham passes to the final labeling operation.

The choice of ingredients to add to brine are a very important step in the determination of ham nutritional and sensorial quality . Brine is considered as a vehicle for the introduction of salts, natural spices, but also polyphosphates, nitrates and nitrites to improves the taste, the water retention and cohesion of the different cuts of meat.

Sugars are also often added to brine: their presence increases cooked ham calorie intake and does not allow it to be consumed by people who have problems of diet and glycaemia.

For this reason, in this type of cooked hams, sugars, and in particular dextrose and fructose are partially or completely replaced by inulin , to reduce the calorie intake. This soluble fiber, retaining water, favors the action of the other additives in the ham ingredients, such as polyphosphates and caseinates. Inulin contributes to give prebiotic properties to ham, as previously mentioned and it has an high capacity to form a viscous mass which slows gastric emptying and consequently absorption of nutrients in the small intestine (Cardellini, 2005).

6.1.3 Aims of this work

The aim of this work was to study the pulsed amperometry detector response in relationship with the molecular weight and the degree of polymerization of carbohydrates. This study was initially applied to maltosaccharides and fructooligosaccharides with commercial standards are available. Then it was extended to FOS and IOS of some commercial fructans like Beneo P95™ and inulin Frutafit IQ.™. Finally two food application are reported regarding quantitative determination of these soluble fiber respectively on prebiotic fermented milks (Chapter 7) and on cooked ham samples.

6.2 Materials and methods

6.2.1 Chemicals

Glucose, fructose and sucrose, were purchased from Sigma–Aldrich (Milan, Italy). Maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose were supplied from Supelco, 1-kestose, nystose and 1-fructofuranosylnystose from Wako Chemicals GmbH (Germany). Beneo P95™ was obtained from Orafti (Belgium).

6.2.2. HPAEC-PAD conditions

For Beneo™ P95 analysis:

-Column: Carbopac PA 100 (4x250 mm) with pre-column (4x50mm). Flow rate:.0.7 mL/min.

For Frutafit IQ ™ analiysis:

-Column: Carbopac PA 200 (3x250 mm) with pre-column (3x50mm). Flow rate:.0.5 mL/min.

6.2.3. Mass spectrometry analysis

An ion trap mass spectrometry equipped with an electrospray interface (6300 Ion Trap, Agilent Technologies, Waldbronn, Germany) was employed for the DP determination.

Carbohydrate are diluted with water: acetonitrile (50:50 v/v) and 0.1% formic acid and detected optimizing these parameters:

-Direct infusion: 10 µl/min

-Negative ion mode

-Capillary voltage:4.1 KV

-Capillary exit voltage: -191V

-Dry temperature: 250°C

-Dry nitrogen gas: 8.0 l/min

-Nitrogen nebulizer pression: 15.0 psi

-Scan range : 170-220 m/z

6.2.4 Cooked ham treatment

Cooked ham samples were prepared by blending two slices of each ham. Ten grams of the homogenized sample were weighed and diluted with 50 mL of HPLC grade water and stirred with a magnetic stirrer. The beaker with the sample was placed in a shaking water-bath at 80°C for 60 min to denature proteins. The sample was centrifuged at 7000 xg for 45 min at 4° C. The clarified solution was removed and an aliquot (1 mL) was diluted with 12 mL of HPLC-grade water (final dilution 1:60). After filtration through a 0.45 μm membrane filter, sample was injected into HPLC.

6.3.Results and discussion

6.3.1. Determination of PAD response of maltooligosaccharides and fructooligosaccharides which standards are commercially available.

Previous studies have reported the PAD response for maltooligosaccharides with low degree of polymerization (DP 2-17), showing that PAD had a different response for chains of different length (Koizumi et al, 1991; Shi and Seib, 1992, Koch et al, 1998). In fact the peak area in a HPAEC chromatogram does not directly reflect the chain length distribution.

Maltooligosaccharides of different DP, corresponding to maltotriose (DP3), maltotetraose (DP4), maltopentaose (DP5), maltohexose (DP6) and maltoheptaose (DP7), are injecting into HPLC at 6 linear and equidistant levels of concentration (range 100÷500 ppm), performing analyses in triplicate. A good linearity ($R^2 > 0.993$) was established for each standard, with differences about the slope of each calibration curve. In fact DP3 and DP4 showed higher slopes values, while for higher DP slopes decreased slightly, mainly from DP4 to DP5 (Figure 2).

The same study was conducted on glucose, fructose, sucrose and on fructooligosaccharides 1-Kestose (DP3), Nystose (DP4) and Fructofuranosyl-nystose. (DP5). As previous observed for maltooligosaccharides, the PAD response decrease with DP increasing. For glucose and fructose slope values (respectively 4.025 and 3.395) are higher than sucrose (1.763), at the same concentration levels of linearity; regarding FOS DP decrease slightly from 1-Kestose (1.618) to Nystose (1.574) and with a great entity for Fructofuranosyl-nystose (1.142). Comparing the slopes between fructooligosaccharides

and maltooligosaccharides it is possible to observe that PAD response is higher for FOS than maltooligosaccharides with the same DP

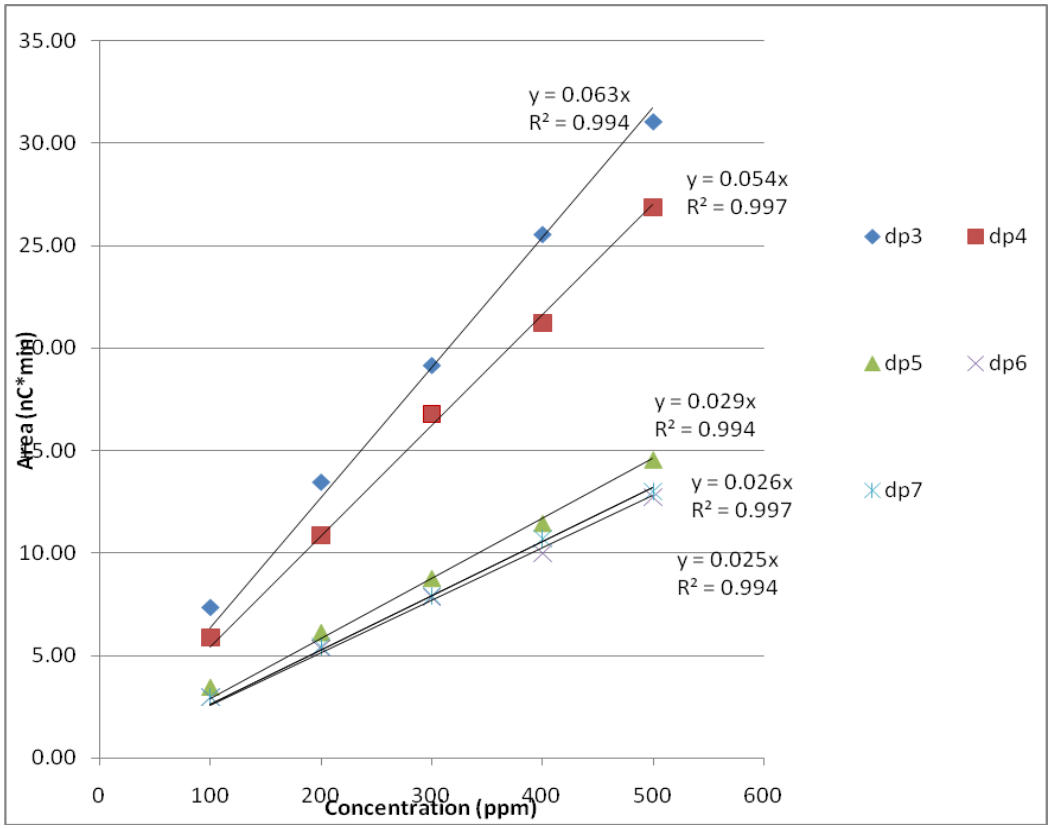


Figure 2. Maltooligosaccharides curves of calibration

Therefore we want to investigate if this difference could be observed also between fructooligosaccharides (formula GFn, as reported in the Chapter 1) and inuloligosaccharides (formula Fn). The main problem associated to Fn and GFn with DP higher than 5 is due to the lack of commercial standards. In this study a commercial mixture of fructooligosaccharides and inulooligosaccharides, named Beneo P95™ was analyzed to verify differences on PAD response.

Considering that producer defines Beneo P95™ as a mixture of simple sugars and oligofructose but the degree of polymerization is not declared, previous analyses by a linear trap mass spectrometer with ESI source are conducted to evaluate the DP distribution of Beneo P95 ™ components. The degree of polymerization of this soluble fiber is 7 but, as previously explained in the Chapter 5, the signals

showed could attribute to inulooligosaccharides or fructooligosaccharides because it is not possible to distinguish FOS and IOS with the same DP (Figure 3).

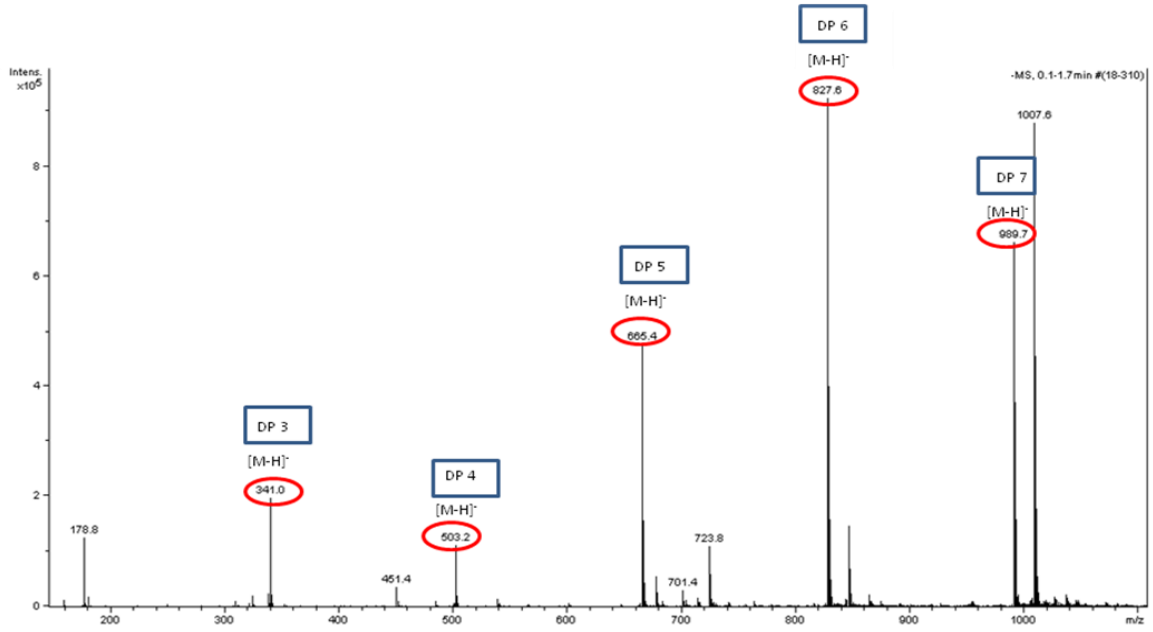


Figure 3-Mass spectrum of Beneo P95™

The use of HPAEC-PAD instead offers the possibility to separate Fn from GFn oligosaccharides, as previously reported (Kang et al, 1999).

Considering that good chromatographic conditions are essential for the study of PAD response and then for reliable quantitative HPAEC analyses, the gradient of elution was optimized.

The best conditions in terms of a good separation, peak resolution and repeatability were found employing a linear gradient of 130 minutes (anticipated by 40 minutes of column conditioning) with an increasing concentration of sodium acetate from 5 to 88 mM and a constant sodium concentration of 60 mM. This conditions had permitted to maintain pH mobile phase constant during the overall gradient elution (LaCourse, 1997).

As showed by the chromatogram (Figure 4), all peaks are separated with a good peak resolution. For the identification of glucose, fructose, sucrose, 1-kestose, nystose and fructofuranosyl-nystose, their retention times are compared with those of available standards.

Regarding distinction between FOS and IOS, we have considered previous works (Kang et al, 1999, Ronkart et al, 2007) that reported that GFn eluted before Fn having the same degree of polymerization. Furthermore the order of elution followed the general accepted assumption that the retention time of a homologous series of carbohydrates increased as the DP increased.

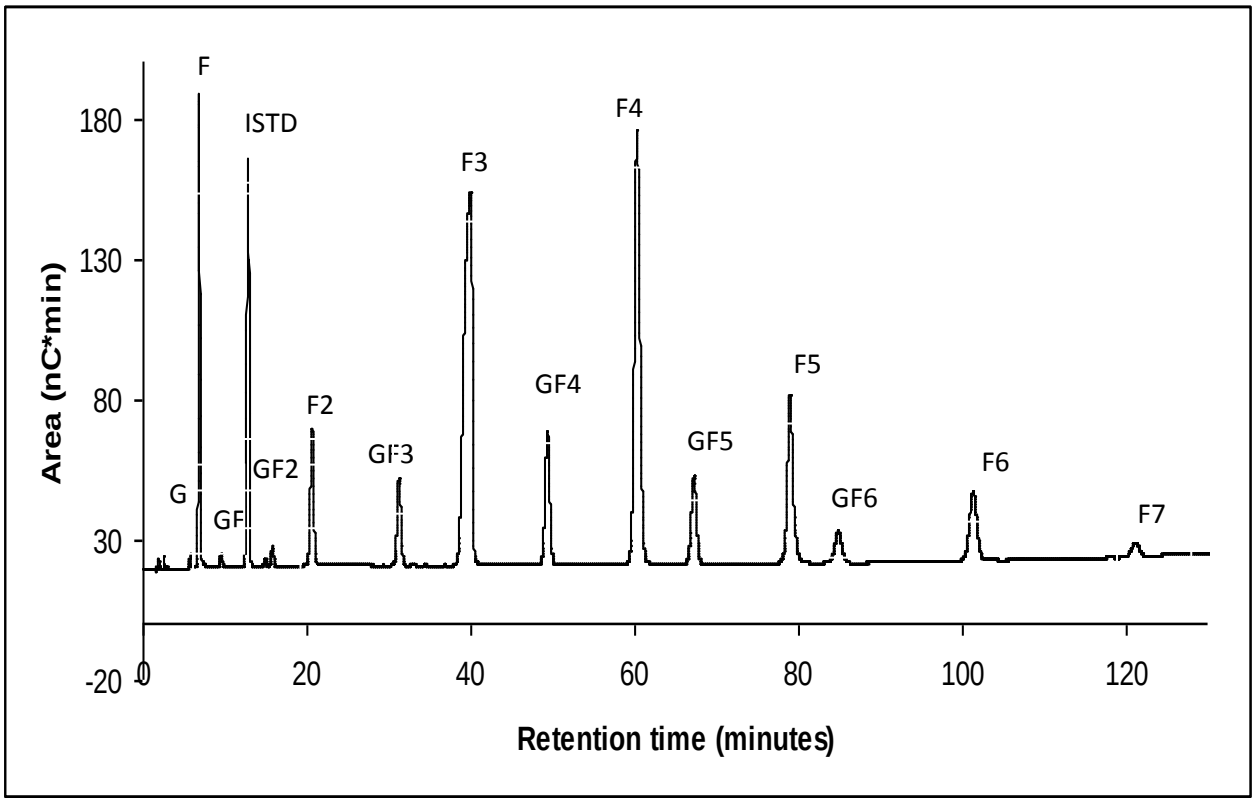


Figure 4: Chromatogram of Beneo P95™.

G=glucose, F=fructose; GF=sucrose; F:inulooligosaccharides: F2=inulo-biose; F3=inulo-triose; F4=inulo-tetraose; F5=inulo-pentaose; F6=inulo-hexaose, F7=inulo-heptaose.

GF: fructooligosaccharides: GF2=1-kestose; GF3=1-nystose;GF4=1-F-1-β-fructofuranosyl-nystose; GF5=1-F-1-(β-fructofuranosyl)-2-nystose; GF6=1-F-3-β-fructofuranosyl-nystose.

ISTD: Melezitose

The order of elution is confirmed by Figure 5 that reported the retention times of fructooligosaccharides and inulooligosaccharides with DP increasing. The linear gradient of elution had

permitted to maintain the same distance between FOS or IOS with DPn and DPn+1. The two regression lines obtained (with R^2 values of 0.999) could help to distinguish Fn series from GFn series and therefore to predict the degree of polymerization in the considered ranges.

With the aim to investigate the variation of detector response for inulooligosaccharides, Beneo P95™ was employed as standard and five equidistant concentration levels were analyzed in the concentration range of 100-500 µg/mL, performing analyses in triplicate (Figure 6). A good linearity was established in the range for each standards with R^2 values ≥0.990. The PAD response decreased at degree of polymerization increasing, as previously observed for fructooligosaccharides and inulooligosaccharides. The slopes values are higher for F3 and F4, then decrease slightly and they seem to be more constant from DP 6.

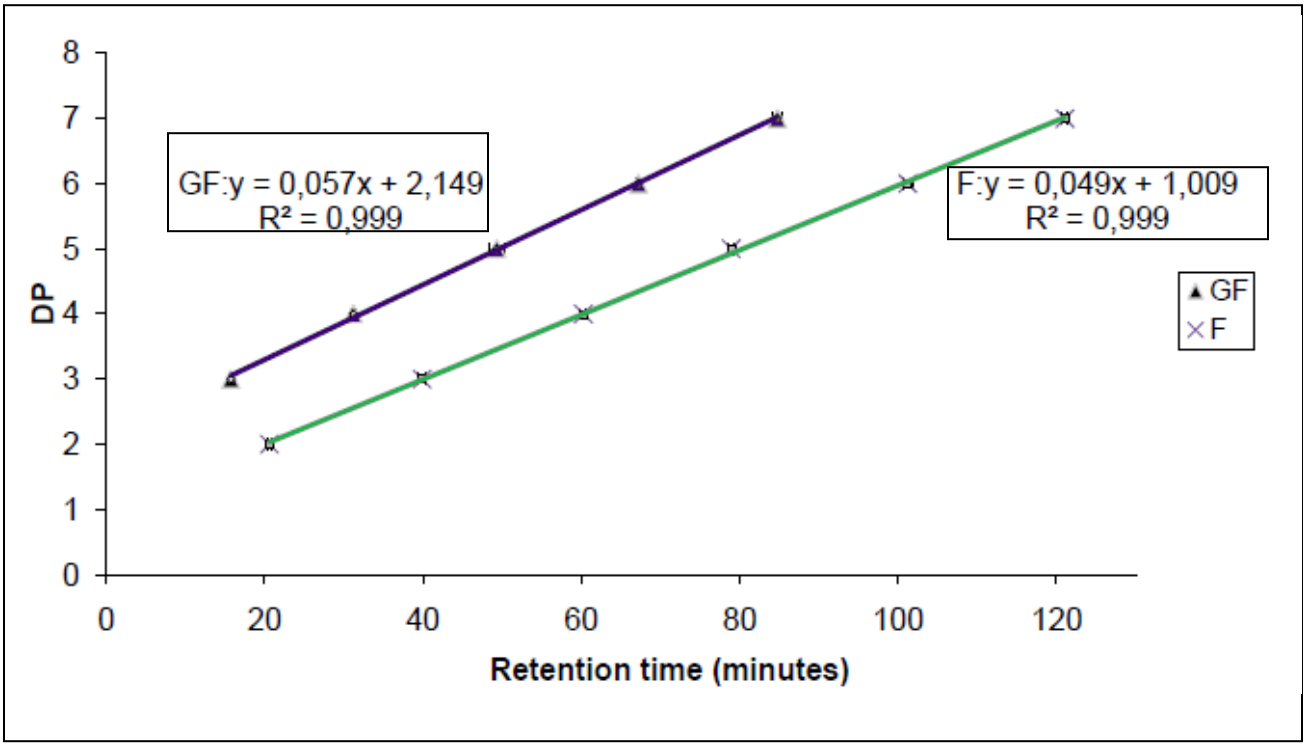
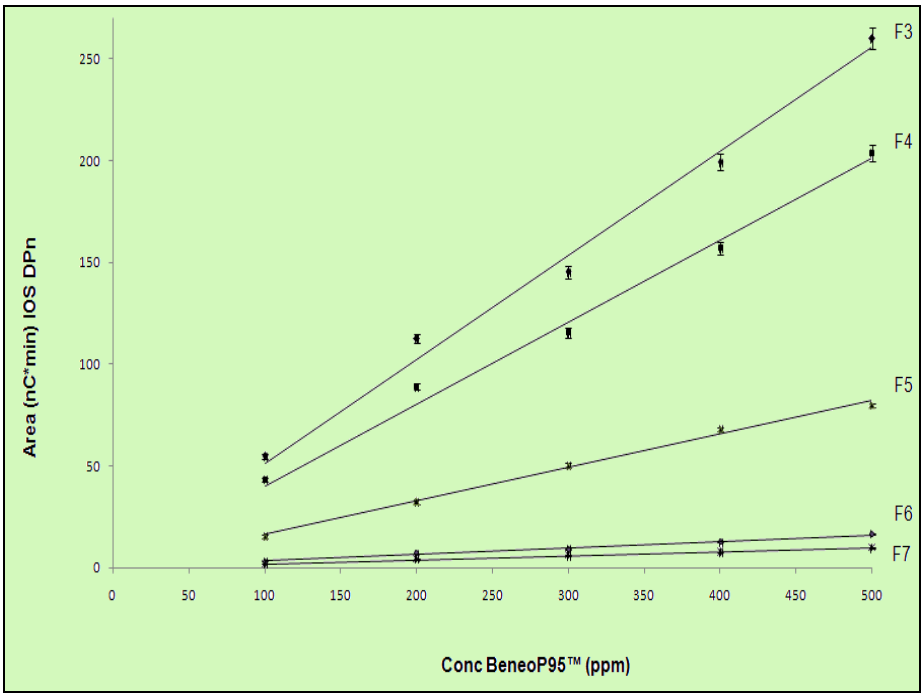


Figure 5-Retention time (min) vs. degree of polymerization for fructooligosaccharides (▲) and inulooligosaccharides (X).



Fn	b	R^2
F3	0.511	0.990
F4	0.402	0.991
F5	0.164	0.994
F6	0.032	0.990
F7	0.019	0.996

Figure 6- Regression curves of inulooligosaccharides in Beneo P95™ and relative slopes in the Table

The slopes of IOS are lower than the co respective DP FOS, as previously observed for maltooligosaccharides. These data suggest that the different effect of each fructose unit on the detector response between IOS and FOS series depends on the presence of reducing activity of oligosaccharides. As the detector measures electrons released from the hydroxyl group of oligosaccharides in high pH condition, the reducing activity of oligosaccharides such as IOS and malto-oligosaccharides series contributes to the access of electrons from ionized hydroxyl groups to the gold electrode of PAD (Kang et al, 1999).

This study was useful for the determination of FOS and IOS in prebiotic fermented milk, as reported in Chapter 7, where our proposal method was compared with AOAC method.

For quantitative determination it was calculated the detector response for each oligosaccharide (both FOS and IOS), adding to Beneo P95 ™ standard and to fermented milk samples an internal standard (melezitose), by this formula:

$$R = \frac{A_{peak} / A_{IS}}{\frac{1}{A_{IS}}\left(\sum_{n=3}^{n=7} A_{scFOS} + \sum_{n=2}^{n=7} A_{IOS}\right)}$$

where:

A_{peak} = area of the eluted oligosaccharide (either from FOS or IOS series);

A_{IS} = area internal standard (melezitose)

$\sum_{n=3}^{n=7} A_{scFOS}$ = total peak area of the eluted fructooligosaccharides

$\sum_{n=2}^{n=7} A_{IOS}$ = total peak area of the eluted inulooligosaccharide series

6.3.2. Determination of PAD response of inulin Frutafit IQ

An investigation of the pulsed amperometry detector was conducted also for an inulin: Frutafit IQ™. An HPAEC-PAD method was optimized, investing the best conditions about column and gradient conditions. The choice of the column is an important parameter for inulins analyses: Carbopac PA100 and Carbopac PA200 are both specific and selective for fructans analyses, but Carbopac PA200 permitted a good resolution for oligomers with an higher degree of polymerization.

In Figure 7 and 8 there are reported two chromatograms of Frutafit IQ ™ obtained with the two different column, with Carbopac PA200 a good separation for all peaks was performed and a total run time was reduced from 120 to 60 minutes. For both analyses a gradient of sodium acetate, the traditional pushing agent for this analyses, was performed, with the advantage to obtained peak well resolved but long-time analyses.

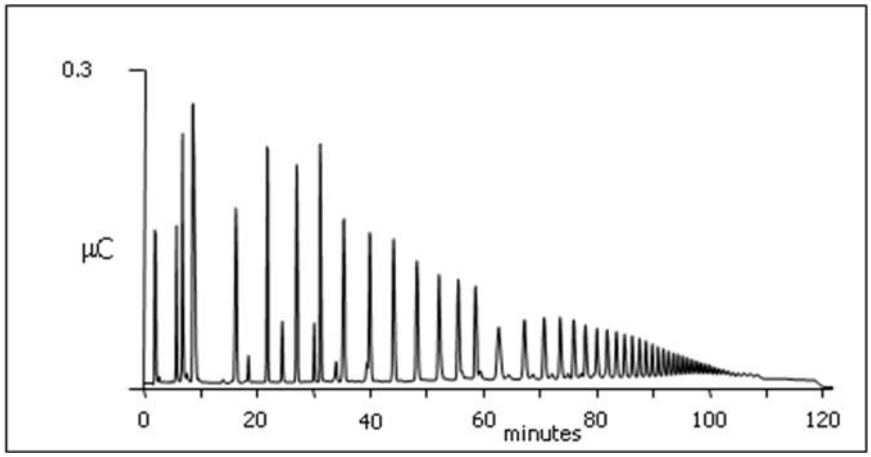


Figure 7. Frutafit IQ™ : Column Carbopac PA100, gradient with sodium acetate

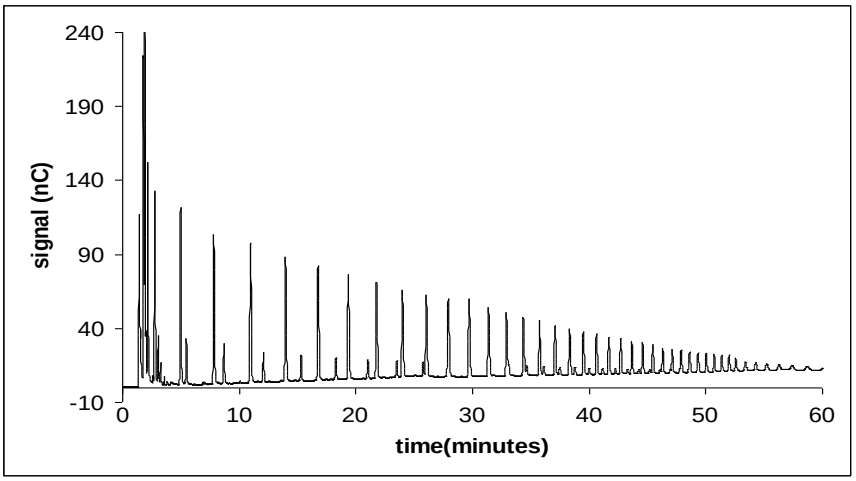


Figure 8-Frutafit IQ™: Column Carbopac PA200, gradient of sodium acetate.

The employment of sodium nitrate instead of acetate as the pushing agent (previously reported by Zhang et al, 1997) had permitted to simultaneous increase of the column peak capacity and the reduction of the analysis time for inulin Frutafit IQ™, as reported in Figure 9. Nitrate have an higher affinity for strong anion-exchange resin then acetate.

This analysis was obtained performed with the Method showed in Table 1.

After optimizing the gradient of elution, it was built a calibration curve for the quantification of the trisaccaride 1-kestose, with the purpose to determine its concentration in the inulin.(Y= 0.218X , R^2=0.997). Therefore a linear relationship between 1-kestose and Frutafit IQ™ concentration, in the linear range from 50-500 mg/L, was found. (Y=0.087X; R^2 =0.996).

For the others peak area, corresponding to fructans with a progressive and increasing degree of polymerization, it was calculated the PAD response factor as ratio of each peak area to 1-kestose area, setting the response factor of this trisaccharide equal to 1.

The response factor was different for each peak, with a generally decrease of these values, but the ratio between each peak area and 1-kestose area resulted constant.

As previously observed for maltooligosaccharides, FOS and IOS, the PAD response decreases at DP increasing, as reported in Figure 9.

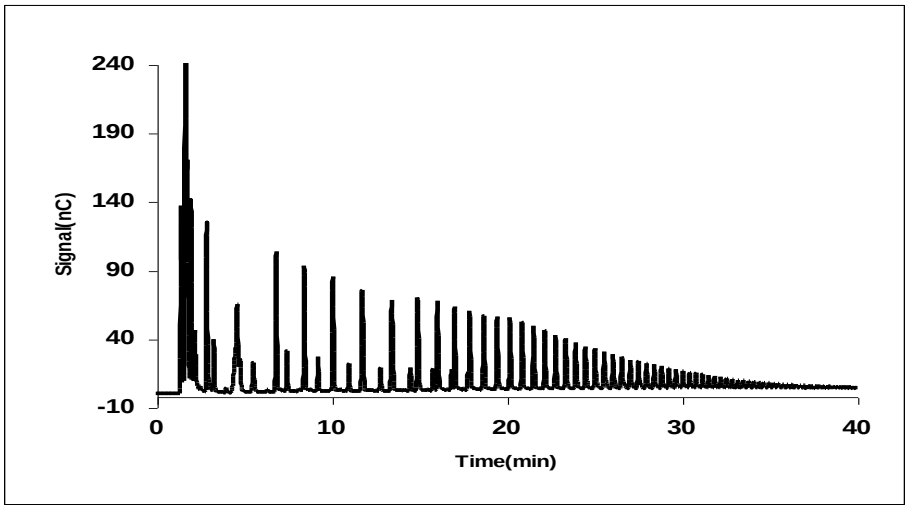


Figure 9. Frutafit IQ: Column Carbopac PA200, gradient of sodium nitrate.

Table 1. Method employed for Frutafit IQ ™ analyses

Elution time (minutes)	A% (H_20 for HPLC)	B% (NaOH, 50% v/v, 600mM)	C% ($NaNO_3$, 250mM)
-40 [a]			0
0	93	7	0
10	93	7	0
40	60	16	24
40.1	0	50	50
50	0	50	50

[a] Negative time indicates time prior injection for the column conditioning.

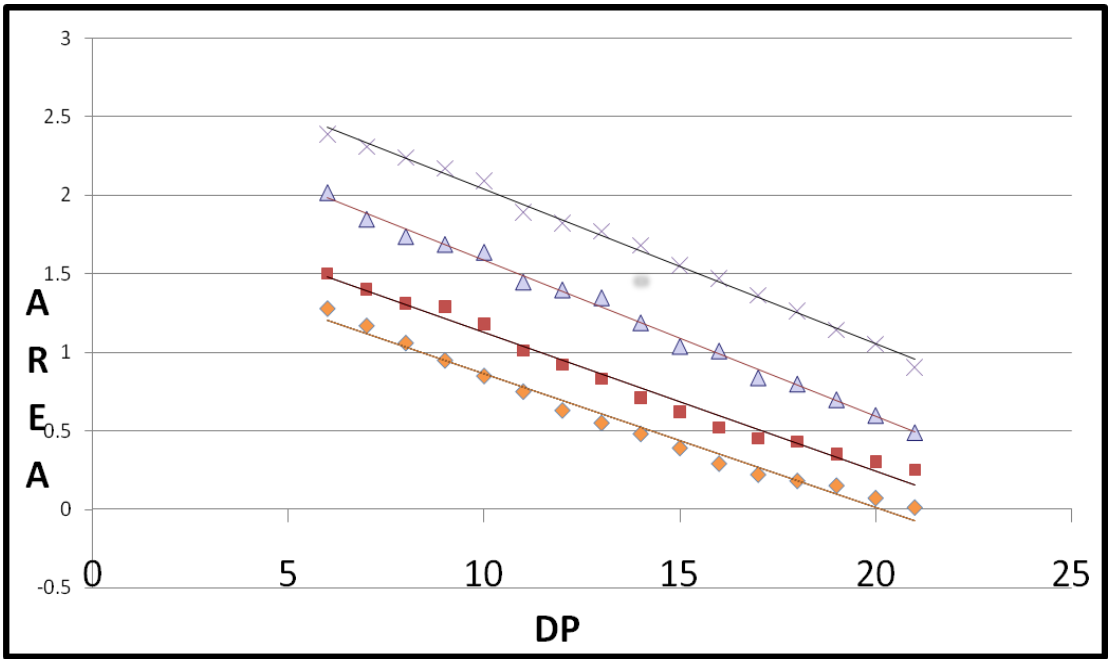


Figure 10- Inulin calibration curves at different concentration ranges (50÷350 mg/l)

It is possible to observe that calibration lines are almost parallel with similar slope values of calibration curves, at different range of concentration , as reported in Table 2.

Table 2. Calibration lines

Frutafit IQ ™ concentration (mg/l)	**Calibration line equations**	**R^2**
50	Y=-0.084X +1.702	0.983
150	Y=-0.088X +2.006	0.984
250	Y=-0.099 X+2.582	0.994
350	Y=-0.122X+3.298	0.995

This study was applied for the determination of Frutafit IQ™ added to cooked ham.

6.3.3. Determination Frutafit IQ in cooked hams.

Inulin was added to the brine used in the industrial process for preparation of commercial cooked ham, prior the cooking step, as a replacer of sugars with the aim of reducing caloric content. The necessity to optimize a method for the quantitative determination of inulin and its components is due to the fact that inulin gel properties and prebiotic characteristic are strictly related to its oligo and polysaccharidic composition and depending on the degree of polymerization (Chiavaro et al, 2007, Corradini et al, 2004).

The method described in 7.3.1. was employed to compare Frutafit IQ™ commercial standard with cooked ham chromatogram (Figure 11).

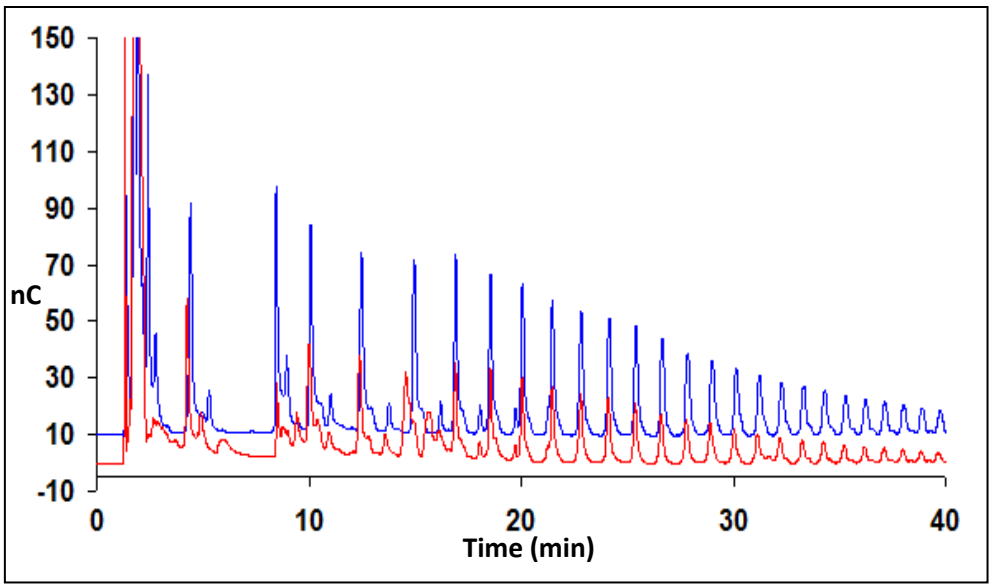


Figure 11.Comparison between chromatographic profile of a Frutafit IQ™ standard solution (blue color) and the chromatographic profile of an extract of inulin from a cooked ham sample. (red colour)

The identification of the inulin was performed by *fingerprinting* the standard chromatograms and the co respective sample chromatogram.

To perform quantitative evaluation of inulin present in cooked ham, we selected six unidentified peaks of the oligosaccharide fraction, which were selectively eluted (Figure 12) by a method than consists in two two-step gradient elution: in the first 5 minutes sodium acetate was increased from 25 mM to 50 mM, then it was increased to 110 mM in 15 minutes, sodium hydroxide was maintained constant to 96 mM. At this 20 minutes of run time followed a step of column washing of 10 minutes and a column conditioning time of 25 minutes.

This method was validated in terms of limit of detection (LOD) and quantitation (LOQ), linearity, precision, and percentage of recovery on a matrix of cooked ham without inulin.

The linearity of response for the selected unidentified peaks was demonstrated at six different concentrations of inulin, ranging from 50 to 300 µg/mL, with coefficient of correlation higher than 0.993 and, as expected, with decreasing slopes values (Table 4).

Recovery percentages were calculated adding three concentration levels (corresponding to 15%, 30% and 5%) of inulin to an ham sample after homogenization (Table 5).

Precision was calculated both as intra-day precision, performing 5 analyses and repeatability on 10 analyses in two non-consecutive days for or each concentration level (75 and 100 µg/mL).Values of precision are reported in Table 6 and are expressed as RSD%.

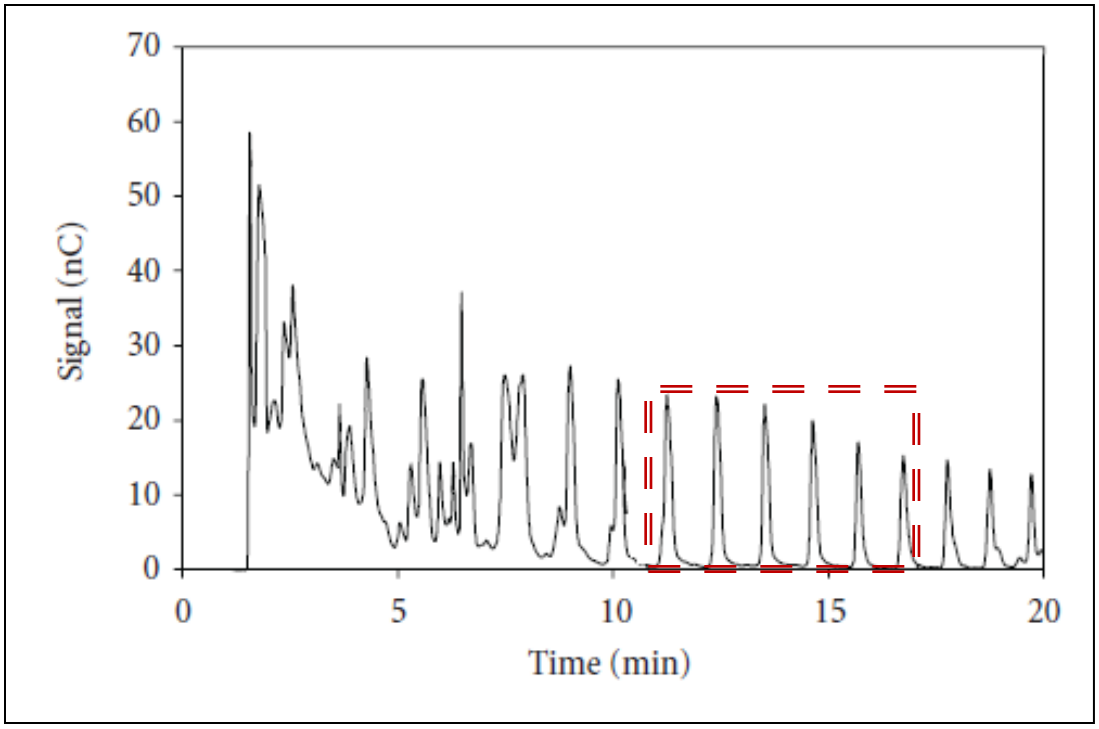


Figure 12. Chromatogram of an extract of inulin from a cooked ham sample, showing in the red box, selected to perform quantitative evaluation of inulin present in the analyzed sample.

Table 3. Limit of detection (LOD) and Limit of quantitation (LOQ)

Peaks	**1**	**2**	**3**	**4**	**5**	**6**
LOD (yD) [a]	12.21	13.65	12.45	13.22	14.27	16.03

LOQ (yQ) [b]	43.32	48.62	49.89	40.67	50.77	51.21

[a] Concentration (μg/g) corresponding to signal and yD=yb+2t (95%, n-1) sb
[b] Concentration (μg/g) corresponding to signal and yQ= yb+10sb

Quantitative determination of inulin was performed on two samples: one cooked on oven at a temperature of 69°C and the other one cooked at 71°C. In particular 10 samples of ham cooked in different oven position at the two temperature are analyzed. As reported in Figure 13, and after a Bonferroni test, no significative differences are showed between samples cooked at 69 and 71°C, considering also a internal variability related probably to the oven position, with an average value of 1.05 ± 0.02 g/100g cooked ham.

Table 4. Linearity

Peaks	**Slopes** (Range: 50-300 μg/ml)	**R^2**
1	0.022	0.998
2	0.021	0.998
3	0.019	0.997
4	0.018	0.997
5	0.016	0.997
6	0.014	0.993

Table 5. Recovery percentages

Peaks	**15%(30µg/ml)**	**30%(60µg/m)**	**45%(90µg/m)**	**Average recoveries (%) (n=3)**		
1	221.27 ± 4.54	242.22 ± 3.19	266.93 ± 2.34	98.55	95.22	93.92
2	209.90 ± 6.95	241.74 ± 4.42	268.37 ± 4.37	102.23	102.79	98.24
3	214.36 ± 2.45	243.49 ± 4.12	266.39 ± 0.37	103.14	98.66	91.10
4	210.48 ± 0.11	250.48 ± 3.75	260.34 ± 0.57	101.50	105.59	97.48
5	191.73 ± 0.94	224.66 ± 0.07	256.64 ± 0.05	101.66	102.85	103.36
6	184.25 ± 5.38	212.27 ± 1.36	245.78 ± 0.10	103.78	102.35	103.60

Table 6. Intraday precision

Peaks	Intraday precision				Repeatability		
	Conc (µg/ml)	**Xm±Sd** [a]	**r** [b]	**RSD(%) (n=5)**	**Xm±Sd** [a]	**Homogeneity of variance (p)**	**RSD (%) (n=10)**
1	75	1.92 ± 0.05	0.17	1.92	1.88 ± 0.12	0.15	2.03
	100	2.14 ± 0.06	0.23	2.93	1.93 ± 0.02	0.57	1.06
2	75	1.87 ± 0.05	0.18	1.69	1.73 ± 0.08	0.22	1.98
	100	1.97 ± 0.05	0.20	3.05	1.79 ± 0.03	0.29	1.88
3	75	1.73 ± 0.03	0.11	1.69	1.68 ± 0.08	0.31	2.98
	100	1.82 ± 0.16	0.16	2.50	1.66 ± 0.03	0.21	1.71
4	75	1.69 ± 0.06	0.06	3.30	1.49 ± 0.09	0.18	2.87
	100	1.68 ± 0.02	0.07	1.20	1.53 ± 0.04	0.08	2.35
5	75	1.55 ± 0.04	0.13	2.33	1.38 ± 0.09	0.19	2.49
	100	1.54 ± 0.11	0.03	1.96	1.42 ± 0.00	0.45	1.18
6	75	1.37 ± 0.04	0.13	2.80	1.15 ± 0.01	0.35	1.85
	100	1.47 ± 0.01	0.05	1.07	1.28 ± 0.03	0.21	2.28

[a] Mean area ± standard deviation

[b] Repeatability limit (95% confidence level) calculated on three measurements.

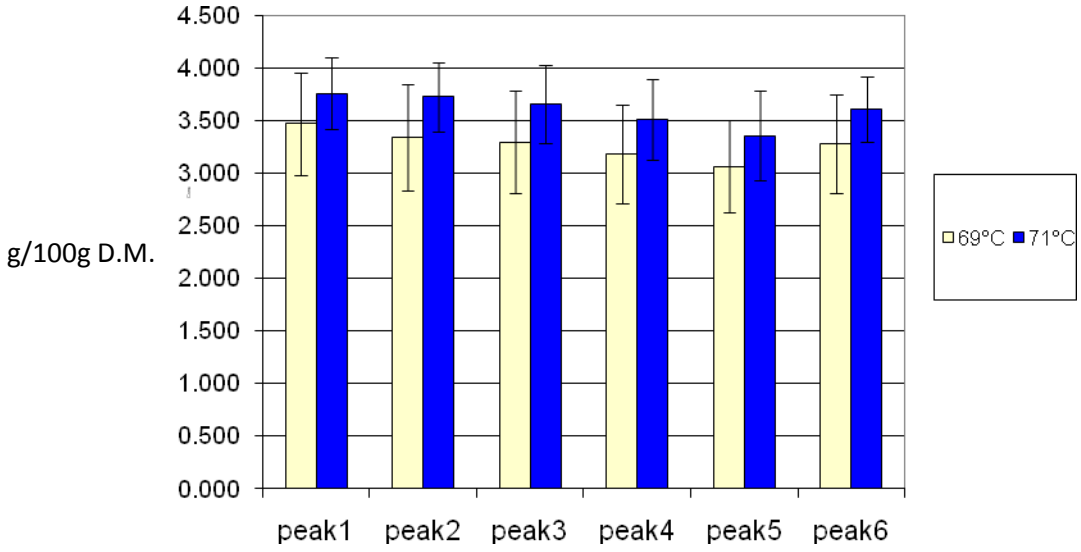


Figure 13. Quantitative analyses of inulin Frutafit IQ™ in ham samples cooked at two different temperatures.

6.4. Ackowledgements

I would thank F.lli Emiliani s.p.a. (Langhirano, Parma) for kindly provinding cooked ham with inulin samples.

6.5. References

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7. DETERMINATION OF FOS AND IOS IN FERMENTED MILKS AND STUDY OF VARIATION OF PREBIOTICS IN RELATIONSHIP WITH PROBIOTICS DURING SYNBIOTIC FERMENTED MILK SHELF-LIFE.

7.1 Introduction

7.1.1 Fermented milks and yogurt as prebiotic foods

Since 1908, when Metchnikoff theorized that fermented milk products provided health benefits, these products are viewed as "healthy" by consumers, and today are perhaps the most employed as prebiotic, probiotic or synbiotic foods. They could define on the basis of the micro-organism responsible for the fermentation in yogurt (Lactobacilli and Streptococci), acidophilus milk (Lactobacilli), yogurt (Lactobacilli and Streptococci), bifidus milk (Bifidobacteria) (Mazza, 1998).

Prebiotics show both important technological characteristics and interesting nutritional properties (Chow, 2002; Huebner et al., 2007). For prebiotics to serve as functional food ingredients, they must be chemically stable to food processing treatments, such as heat, low pH, and Maillard reaction conditions. That is, a prebiotic would no longer provide selective stimulation of beneficial microorganisms if the prebiotic was degraded to its component mono- and disaccharides or chemically altered so that it was unavailable for bacterial metabolism. (Wang, 2009).

Fermented milks and yogurt are often employed as functional foods by adding prebiotic ingredients. In a recent work, Guggisberg et al, 2008, studied the reological, microstructural and sensory characterization of a low-fat yoghurt with inulin addition. They observed an higher yield stress, "firmness" and "creaminess" values in yoghurt with higher inulin additions.

7.1.2. Synbiotics: Definition and State-of-art.

A very promising area in the development of enhanced functional food ingredients is the development of synbiotics.

Synbiotics are defined as a combination of a probiotic and a prebiotic (Gibson and Roberfroid, 1995); there has been a lot of recent interest in the concept. Because the word alludes to synergism, this term

should be reserved for products in which the prebiotic compound selectively favors the probiotic compound.

There are vary types of oligosaccharides commercially produced, all claimed to be bifidogenic by the manufacturers and they include fructooligosaccharides (FOS), galactooligosaccharides (GOS) and transgalactosyloligosaccharides (TOS), and soybean oligosaccharides (main functional components being raffinose and stachyose), that have been most extensively studied and may provide the best evidence of prebiotic effects in humans (Crittenden 1999; Crittenden and Playne 1996).

Shin et al, 2000, investigated the degree of enhancement of growth, activity and viability of *Bifidobacterium* Bf-1 and Bf-6 in skim milk in the presence of FOS, GOS, and inulin and they found that their viability was great in the presence of FOS followed in a descending order by GOS and inulin. The effects of oligosaccharides and inulin increased with increasing carbohydrate concentration and were maximal at 5% (w/v).

More recently Hubner et al, 2007, conducted a similar study to select which prebiotics (fructooligosaccharides, inulin, and galactooligosaccharides) support selective growth of *lactobacilli* and *bifidobacteria*. By means of the calculation of a prebiotic activity score, they provide a basis for evaluating possible synbiotic food application because only certain combinations between probiotics and prebiotics gave high score (for example Lactobacillus paracasei 1195 grown on inulin). In this strict sense, a product containing oligofructose and probiotic bifidobacteria would fulfill the definition, whereas a product containing oligofructose and a probiotic *Lactobacillus casei* strain would not. However, one might argue that synergism is attained in vivo by ingestion of lactobacilli on the one hand and promotion of indigenous bifidobacteria on the other hand (Schrezenmeir J. and de Vrese M., 2001).

The major strains of bacteria used in probiotics are Lactic acid bacteria (LAB) like *Lactobacillus acidophilus* and various *Bifidobacterium* spp., that are dominant organisms in human small and large intestines, respectively. These micro-organisms play a role in inhibiting the growth of pathogenic organisms through production of organic acids and bacteriocins and by deconjugation of bile salts. The consumption of probiotics has the potential to aid lactose digestion (Vesa et al, 1996), to prevent traveler's diarrhea (Oksanen et al, 1990), to reduce the duration of rotavirus diarrhea (Guarino et al,

1998), to exert antitumor activity (Kato et al, 1994), to enhance the activity of the immune system (Meydani et HA, 2000) and to aid in controlling serum cholesterol (Gilliland et al, 1985).

The prevalence of these organisms in the intestines may be reduces with age, dietary changes, antibiotic consumption and/or stress, and their absence of low viability may cause varying degrees of digestive problems. (Mazza, 1998).

Kurmann and Rasic, 1991, recommended that the minimum dose of probiotic able to assure therapeutic effect should be in a range between 8 and 9 log cfu/mL.

In Japan, the Fermented Milks and Lactic Acid Beverages Association has already established a standard that requires 7 log cfu/mL to be present in dairy products that claim to contain bifidobacteria where the Swiss Food Regulation as well as International Standard FIL/IDF requires that such products contain 6 log cfu/mL of bifidobacteria (Ishibashi and Shimamura,1993). Other researchers suggested counts in range 7-8 log cfu/ml (Dave and Shah, 1997). It is important, to produce the desired effects that probiotic bacteria are present in the product in viable counts during their whole shelf-life. It seems reasonable to assume that the beneficial effects of *L.acidophilus* and *Bifidobacterium* can be expected only when ingesting viable cells which they can colonize the human gut . Survival of these microorganisms could be affected by low pH of the environment (Shah, N, P et al, 1995).

Probiotic bacteria grow slowly in milk because of the lack of proteolytic activity (Klaver et al, 1993). The usual practice is to add conventional yoghurt bacteria such as *Lactobacillus delbrueckii subsp. bulgaricus* and *Streptococcus thermophilus* to reduce fermentation time in probiotic yoghurt manufacture (Dave and Shah, 1998,a). However *L. delbrueckii subsp. bulgaricus* also produces lactic acid during refrigerated storage, which is claimed to affect the viability of probiotic bacteria (Dave et Shah, 1997). To overcome this, it is usually to use starter cultures that are devoid of *L. delbrueckii subsp. bulgaricus* (Dave and Shah, 1998b). Thus, when formulating mixed starters, it is important to study the antagonistic and synergistic interaction between strains, in order to select to those pairs in which a protocooperative effect is observed. (Moreira et al, 2000).

Furthermore the incorporation of micronutrients to the milk, such as peptides or amino acids, may be useful to reduce fermentation time. For example milk protein derived from the fractionation of milk and dairy products, improve yoghurt texture (Güzman-Gonzales, Morais, Amigo, 2000). Oliveira et al,

2001, showed the influence of milk supplementation (whey, caseinate, hydrolysate, milk proteins) in the texture of fermented milks , while they weakly affected the stability of probiotic bacteria.

The rate of fermentation of oligofructose by pure cultures of several species of bifidobacteria is comparable to that of glucose (Roberfroid et al, 1998).

Oligosaccharides are able to stimulate bifidobacterial growth in continuous chemostat cultures inoculated with faeces (Gibson and Wang, 1994) .

It seems that bifidobacteria can utilize these substrates owing to the production of enzymes towards the fructosyl β (2-1) linkages. β –fructofuranosidase (EC 3.2.1.26) can be classified upon their substances specificity as levanes, inulinases or invertases, although many of these enzymes are capable of hydrolyzing more than one type of substrate, therefore being designated as unspecific β- fructofuranosidase. Janer et al, 2004, studied the ability of β –fructofuranosidase from *Bifidobacterium lactis* DM 10140 to cleave a varieties of fructooligosaccharides and inulin and they found that the growth of *B.lactis* was supported by fructans of a low degree of polymerization, as Raftilose™ and Raftiline™ LS, but not with highly polymerized inulin, as Raftiline ™ HP.

7.1.3. Aim of this work

The principal aim of this work was to optimize a method to quantify fructooligosaccharide and inulooligosaccharide fraction of two commercial soluble fibers, Actilight™ and Beneo P95™ added respectively to prebiotic and a synbiotic fermented milks. This method was compared with the AOAC enzymatic method and it was applied to study the prebiotic variation in the synbiotic fermented milks induced by the probiotics *(Bifidobacterium lactis and Lactobacillus acidophilus)* during forty days of *shelf-life*.

7.2 Materials and method

7.2.1 Commercial standard

Beneo P95™ and Actilight ™ were respectively supplied by Orafti (Tienen, Belgium) and Actilight by Beghin Meiji.1-kestose (purity 99.7%), nystose (purity 99.8%) and 1F –Fructofuranosylnystose (purity 86.5%) fructooligosaccharides standard are purchased by Wako Chemical GmbH (Neuss, Germany) while glucose, fructose, melezitose and lactose by Sigma. All stock solutions were prepared at 10 mg/mL with HPLC-grade water and filtered on a 0.45 mm membrane filter.

7.2.2. Samples

Low-fat (0.1%) fermented milks analyzed are prepared by Parmalat (Parma, Italy). The first ones are prebiotic commercial fermented milks with Actilight by Beghin Meiji (Thumeries, France), the second ones are prepared on scale up in this following mode:

-"SFYP" was a fermented milk with only probiotics addition (*Bifidobacterium lactis* and *Lactobacillus acidophilus*)

-"FY" consisted in a prebiotic milk: the oligosaccharide Beneo ™ P95 was added in the amount of 2.20 g/100g of product.

-"FYP" was a synbiotic milks with the presence of both prebiotics and probiotics.

Milk proteins are added to all these fermented milks to have a final quantity in the final product of 4.8%.

-"SFY", "FY" and "FYP" are analyzed during fermented milk *shelf-life* (40 days) each 10 days and from samples production.

7.2.3. Samples treatment

One gram of fermented milk was accurately weighted and diluted with HPLC grade water in a ratio 1:50. An aliquot of the sample corresponding to 4 mL was put in two ultrafiltration spin column with a membrane of 10.000 MWCO (Sartorius, Goettingen, Germany), and centrifuged for 30 min at 8000

rpm with the purpose to purify fermented milks from proteins and lipids. All samples were stored at 4°C and first injection were filtered through a 0.45 μm membrane filter.

7.2.4. Enzymatic determination

Enzymatic analysis was performed in agreement with the method optimized by Andersen and Sørensen, 1999. This procedure consists in a previous extraction of the sample corresponding to approximately 1 g of fructans with boiling water (this fraction is named "E0") and subsequently in a hydrolysis using α-amyloglucosidase solution (200 units/mL Megazyme, Bray, Wicklow, Ireland), corresponding to fraction named E1. A part of the hydrolysate is then treated with fructozyme (Novozymes, Sigma), and this fraction is named "E2". "E0", "E1" and "E2" are firstly diluted, then ultracentrifuged and filtered, first the injection in HPAEC-PAD. Calculation are the following: free glucose, free fructose and sucrose are determined in the fraction "E0," while the sum of the amounts of free glucose, glucose from starch and maltodextrins is determined in the fraction "E1". In our samples no starch or maltodextrins are declared in the labels or added during industrial process. The total amount of glucose and fructose are determined in the fraction E2. Finally, the concentration of glucose and fructose released from fructans is calculated by the differences from these determinations.

7.2.5 Enumeration of Streptoccocus thermophilus and Lactobacillus bulgaricus and probiotic bacteria

Streptococcus thermophilus and *Lactobacillus bulgaricus* were counted following ISO 7889:2003 (Yogurt -- Enumeration of characteristic microorganisms -- Colony-count technique at 37 degrees C). Thermophilic Streptococci were counted in M17 agar after incubation at 37° C for 72 h., while *Lactobacillus bulgaricus* were plated on MRS agar plates acidified at pH= 5.4 and plate counts were determined after anaerobic incubation for 72 h at 37°C.

Regarding probiotic counts, *Lactobacillus acidophilus* were counted following an internal Parmalat method analysis in agree with Vinderola and Reinhemer, 2000. MRS agar was additionated with 0.1% of sodium taurocholate with the aim to inhibit the growth and the count of *Lactobacillus bulgaricus*

because its presence could be interfere in *Lactobacillus acidophilus* count. Plates were incubated at 37° C for 72 h.

Bifidobacterium lactis were counted following an internal Parmalat method analysis in agree with Shah, 1997 and the Bulletin of the IDF 340 (Guideline for the Enumeration of Bifidobacteria in fermented dairy products, IDF Group E104-Lactic acid bacteria and starters). The MRS agar was additionated of lithium cloride, L-cystein cloride, sodium propionate, with the purpose to inhibit others lactic bacteria in the samples. After plates anaerobic incubation at 37° C for five days, plates count were determined. All colonies are counted and expressed as Log cfu/g.

7.3. Results and discussion

7.3.1. Qualitative evaluation of prebiotic fermented milks chromatographic profiles

In the Chapter 6 it is reported the optimization of HPAEC-PAD method regarding the study of PAD response and for analysis of the commercial standard Beneo P95™.

This linear gradient of 130 minutes had permitted to evaluate chromatographic profile of fermented milks with respectively addition of Actilight ™ and Beneo P95™ (Figure 1).

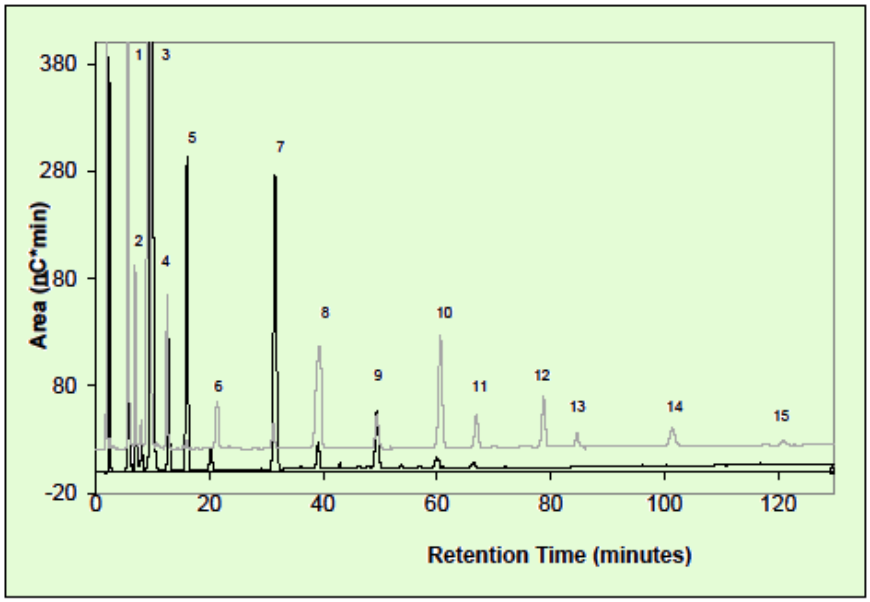

Figure 1: In grey: Fermented milk with Beneo P95™ In black: Fermented milk with Actilight™ Glucose, 2:Fructose; 3: Sucrose; 4: Lactose; 5:GF2; 6:F2; 7:GF3; 8:F3; 9:GF4; 10 F4; 11:GF5; 12:F5; 13:GF6; 14:F6, 15:F7.

Differences about these oligosaccharides are not only about the degree of polymerization that, as previously confirmed by Mass Spectrometer analyses (Chapter 6-7), is respectively 5 for Actilight™ and 7 for Beneo™ P95 , but also for the relative ratio between fructooligosaccharides and inulooligosaccharides series. In these soluble fibers both series are present but while in Actilight ™ FOS are dominant on IOS, mainly GF2 and GF3, in Beneo ™ P95 inulooligosaccharides peaks have an higher signal and area than the co-respective DP fructooligosaccharides. Actilight ™ is industrially produced through fructosyl transfer from sucrose using a fungal enzyme , while Beneo™ P95 is a natural ingredient extracted from chicory root after purification and endoinulinase hydrolysis .These differences related to the technological process of production could influence on the relative ratios between FOS and IOS and in this case, determination of GF and F could be useful to distinguish fructans derived from natural sources from fructans industrially produced.

7.3.2 Determination of sugars and commercially available FOS in fermented milks

Quantitative analyses are conducted initially on glucose, fructose, sucrose and on fructooligosaccharides with DP ranging 3-5, commercially available, then on the others FOS and IOS and finally on the total of oligosaccharides.

Initially limit of detection and quantitation of glucose, fructose, sucrose, 1-kestose, nystose and fructofuranosyl-nystose are determinate according to the following formulas:

LOD: 3sb/b

LOQ: 10sb/b

where sb was the SD calculated on ten measurements and b was the slope of calibration curve built on a linear range different for each standards and reported in the Table 1.

Then quantitation of glucose, fructose, sucrose and fructooligosaccharides with DP ranging 3-5 was performed in a linear range between 25–125 mg/mL, injecting three replicated.

A good linearity was demonstrated for glucose (b=4.025; R^2=0.995), fructose (b=3.395; R^2=0.991), sucrose (b=1.763; R^2=0.992), 1-kestose (b=1.618; R^2=0.986), nystose (b=1.574; R^2=0.993), fructofuranosyl-nystose (b=1.142; R^2=0.996).

Table 1. Values of Limit of Detection and Limit of Quantitation

	Range (µg/mL)	**Slopes (b)**	**R^2**	**LOD (µg/mL)**	**LOQ (µg/mL)**
Glucose	0.06-0.86	4.412	0.997	0.09	0.31
Fructose	0.06-0.86	3.763	0.997	0.13	0.43
Sucrose	0.20-1.80	2.102	0.999	0.41	1.36
1-Kestose	0.10-1.30	1.971	0.996	0.19	0.63
Nystose	0.16-1.36	1.812	0.993	0.33	1.09
Fructofuranosyl-nystose	0.25-1.85	1.279	0.996	0.51	1.71

LOD calculated as: 3sb/b
LOQ calculates as 10sb/b

Results of quantitative analyses are resumed in Table 2 as they are reported as an average on a dry matter. They confirmed as previous observed in qualitative analysis: in Beneo P95™ FOS are in a small quantities, in fact the sum of GF2, GF3 and GF4 is lower than 6% and the total amount of free carbohydrates (glucose, fructose and sucrose) is about 7.5% while in Actilight ™ FOS are in considerable amount (about 76% w/w). In Actilight ™ GF2 resulted the principal FOS, followed by GF3 and in a small percentage GF4.

Table 2. Values of FOS GF2, GF3 and GF4 in two fermented milk samples

FOS	Beneo P95™ Mean value (mg/g fermented milk) ±Sd; (g/100g fiber)	Actilight ™ Mean value (mg/g fermented milk) ±Sd; (g/100g fiber)
1-Kestose	0.12±0.01 (0.41)	7.52±0.21 (46.58)
Nystose	0.51±0.01 (2.37)	6.25±0.19 (38.82)
Fructofuranosyl-nystose	0.62±0.02 (2.84)	1.25±0.05 (7.83)

7.*3.3 Determination of FOS and IOS, which standard are not commercially available, in fermented milks.*

Considering that only the 13.5% of carbohydrates was possible to determine in prebiotic fermented milks with the employment of commercial available standards, it was necessary to perform a method for the quantification of FOS with DP>5 and IOS in DP ranging 2-7.

This method was optimized after an accurate study of the PAD response reported in Chapter 6 and it consists in building linear regression of the detector response for each IOS and FOS oligosaccharides versus prebiotic product concentration.

The results of fructooligosaccharides and inulooligosaccharides percentages in the two fermented milks, are reported in Table 3 (Actilight ™ product) and in Table 4 (Beneo P95 ™ product).

In Actilight ™ the total FOS was 77.77%, while in Beneo P95 ™ the total FOS was only 13.32%.

In order to evaluate the accuracy of this method, recovery was determined by adding three percentages of prebiotics (Actilight ™ or Beneo P95™), 20, 40 and 60% of the concentration of the matrix, to fermented milk samples. Percentages of recovery resulted in the range from 92.9 to 93.6% with RSD% <1.5.

Table 3. Percentage of GF and F determined in fermented milks on total Actilight ™ fiber calculated by the formula $(DP_n / \sum nDP) * 100$ (Chapter 6)

Actilight™	DP	b	R^2	%
GF	2	2.24	0.997	43.92
	3	3.21	0.992	29.01
	4	25.52	0.992	4.84
F	2	19.05	0.993	8.59
	3	22.05	0.996	5.97
	4	29.14	0.992	3.89

Table 4. Percentage of GF and F determined in fermented milks on total Beneo P95 ™ fiber calculated by the formula $(DP_n / \sum nDP) * 100$ (Chapter 6).

Beneo P95 ™	DP	b	R^2	%
GF	2	78.67	0.998	0.41
	3	30.24	0.998	2.43
	4	28.78	0.994	2.81
	5	16.31	0.991	6.27
	6	47.24	0.998	1.40
F	2	21.03	0.990	5.81
	3	2.99	0.999	35.47
	4	3.80	0.999	26.57
	5	9.29	0,992	12.07
	6	17.96	0.998	4.97
	7	38.67	0.995	1.89

7.3.4 Determination of the total soluble fiber by the comparison with AOAC method

The results obtained from the optimized quantitative method are compared with those from the official enzymatic method (Andersen and Sørensen, 1999) in order to evaluate the reliability of the proposed method.

In our method we calculated the total content as sum of IOS and FOS area, building a calibration curve for Beneo P95 ™ (b=1.540, R^2=0.994) and for Actilight ™ (b=0.628; R^2=0.993) to express results as g/100g fermented milk. Beneo P95 ™ and Actilight ™ resulted respectively 2.11±0.02 and 1.62±0.03 g/100g fermented milk, in agreement with those reported in the label of one of the two investigated fermented milk, which is commercially available.

Results obtained from AOAC method are not significatively different: in fact Beneo P95 ™ resulted in a concentration of 2.15±0.05 g/100g fermented milk and Actilight ™ in a concentration of 1.61±0.06 g/100g fermented milk.

The advantage of our method is related to the fact that not only the total content fiber can be determine but also the prebiotic effectiveness of FOS and IOS of different degrees of polymerization could be evaluated monitoring the changes in their molecular weight distribution during the *shelf-life* in a synbiotic fermented milk in relationship with probiotic activity.

7.3.5 Determination of Prebiotics in Synbiotic Fermented Milks during shelf-life

The synbiotic fermented milk with addition of Beneo P95 ™ and probiotics ((*Bifidobacterium lactis* and *Lactobacillus acidophilus*) was monitoring during 40 days of product commercial *shelf-life*. To reach this goal an HPAEC-PAD method was optimized by the modification of the previous linear gradient method to reduce analysis time. The column Carbopac PA200 was employed in substitution of Carbopac PA100 and the sodium nitrate as pushing agent was preferred to sodium acetate, obtaining the total elution of FOS and IOS in only 25 minutes.

This method consists in two step of gradient: in the first sodium hydroxide was increased from 60mM to 90mM in the first 4 minutes and in the second step sodium nitrate concentration was increased from 2.5 mM to 15mM in 21 minutes and sodium hydroxide was maintained constant. The column was then washed and equilibrated for 40 minutes in the initial conditions.

The chromatographic profile obtained under this condition (Fig. 2) is similar to which obtained by the previous method, with a prevalence of inulooligosaccharides on fructooligosaccharides and a DP of 7.

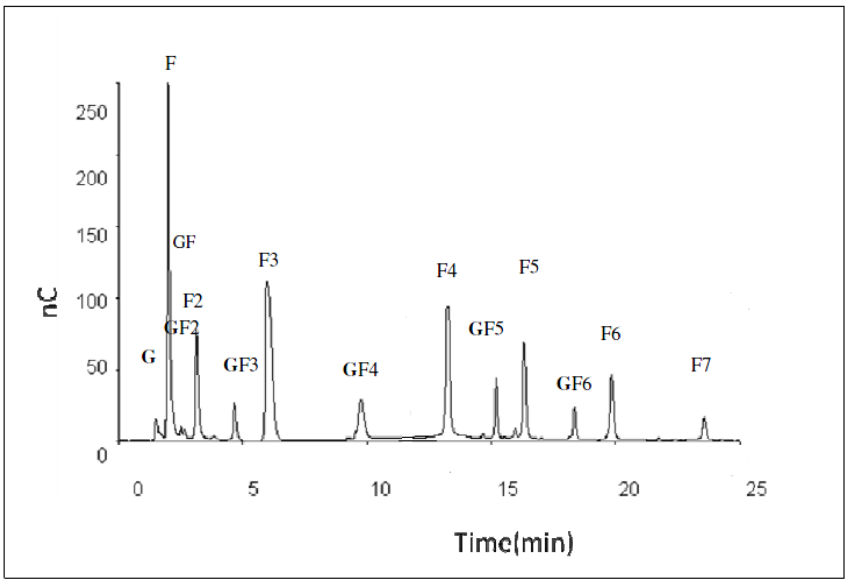

Figure 2-Chromatografic profile of Beneo P95 ™ (Column: Carbopac PA200)

To monitor eventual changes on oligosaccharides fraction of synbiotic milks during 40 days of storage, we choose to quantify the last 6 peaks of the chromatogram corresponding to FOS with DP5-6 and IOS from DP 4 to DP7. This choice is due to the lack of information about the variations of oligosaccharides at medium-high DP in relationship with probiotics.

This method was validated, in accord to Feinberg et al, 2009, which has recently proposed a full validation of HPAEC-PAD method for fructooligosaccharides determination.

Quality parameters were investigated on a matrix of fermented milk (sample named "SFYP").

Limit of detection and quantitation are calculated following EURACHEM guidelines as respectively yD=yb+2t (95%, n-1)sb and yQ=yb+10sb and building calibration curves in a specific range of concentration to convert the signal domain to concentration. Values obtained for GF5 and GF6 and for F series from F5 to F7 are reported in Table 5.

Table 5. LOD and LOQ values

Oligosaccharides	LOD (µg/g)	LOQ (µg/g)
F4	8.33	67.22
GF5	10.56	93.33
F5	11.89	93.89
GF6	15.22	86.67
F6	11.27	72.77
F7	15.68	98.19

Linearity was determined in a linear range from 100 to 500 mg/L, performing analyses in triplicate for each level, with R^2 values in a range from 0.988 to 0.995 (n=15).

Precision was calculated in terms of intra-day precision, performing analyses in the same day and in terms of repeatability (in two non-consecutive days) at two levels of concentration. Values of intra-day precision resulted in the range from 0.46 to 0.90% in term of area RSD% while RSD% values of repeatability were in the range from 0.09 to 0.54%.

Recovery percentages are calculated as (Cf-C0/a *100) , where Cf= concentration determined in the fortified sample Co=concentration determined in unfortified sample and a=concentration of fortification, adding Beneo P95™ to sample at three different concentration (corresponding to 10, 20 and 30%) in triplicate. Average recoveries percentages resulted in the range from 98.31 to 99.16%.

Calibration curves built for the determination of linearity were also employed for the quantification of FOS and IOS in synbiotic fermented milk.

Variation on the fructooligosaccharides and inulooligosaccharides are monitoring both on FY sample where probiotics are not added (Figure 3) and on FYP synbiotic sample (Figure 4).

A Bonferroni test was conducted on the concentration of all oligosaccharides concentration determined during 40 days of fermented milk storage. As showed by Figures 3-4, fructooligosaccharides and inulooligosaccharides decreased in both fermented milks during products *shelf-life,* mainly in the first

ten days , as previous observed by Corradini et al, 2000, that marked that this decrease was mainly from the 8th and 10 th days of storage at 4°C.

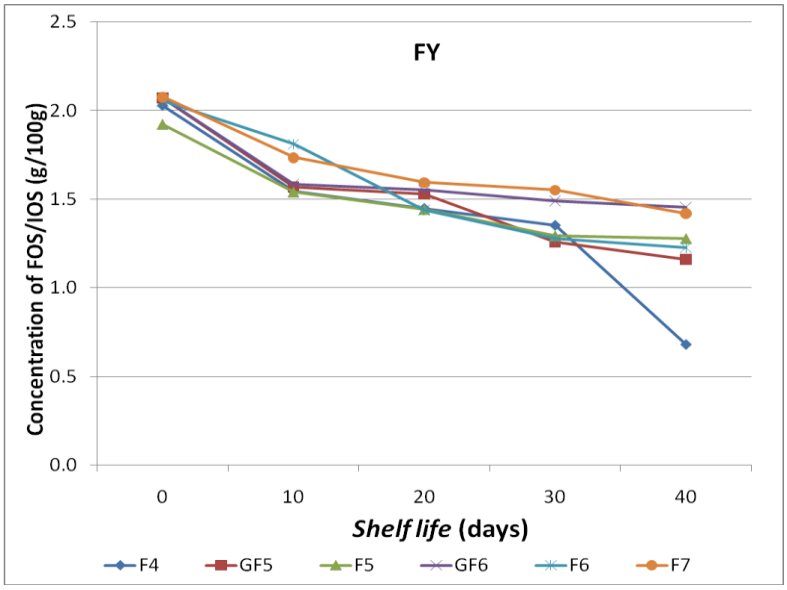

Figure 3- Variations of the FOS and IOS during prebiotic fermented milk (FY) *shelf life*

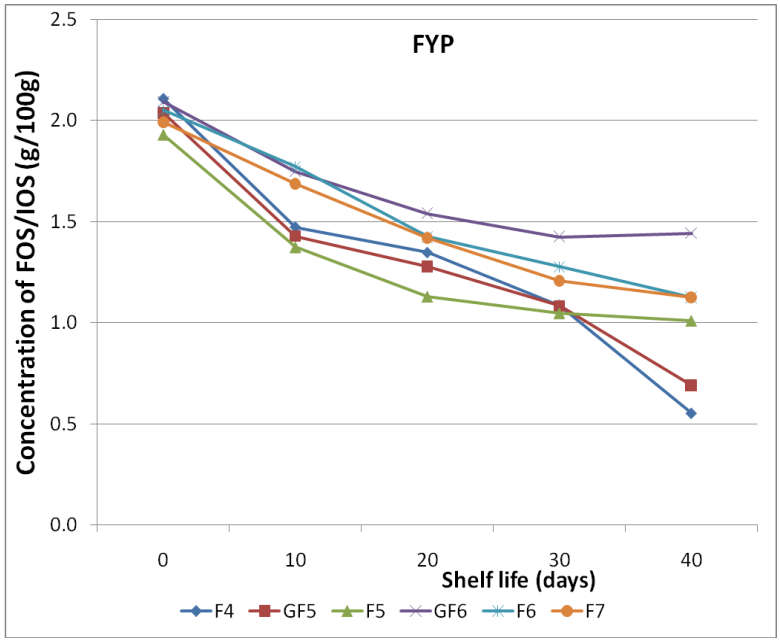

Figure 4- Variations of the FOS and IOS during synbiotic fermented milk (FYP) *shelf life*

Oligosaccharides with lower DP, as for example F4, showed a more marked decrease (correspond to a percent decrease rate of 62%) than oligosaccharides with higher DP, as F7(correspond to a percent decrease rate of 43%). These variations are observed also in FY sample, but in a lower entity. These differences between oligosaccharides with different degree of polymerization could be attribute to the fact that *Bifidobacterium* strains has an higher capability to degrade fructans with a lower DP , as previously reported by Rossi et al, 2005. Regarding microorganism with no probiotic properties, *Lactobacillus bulgaricus* strains seem to be capable to metabolize fructans (Kaplan and Hutkins, 2000), while *Streptoccocus thermophilus* is a fructooligosaccharides-nonfermenter.

7.3.6 Determination of Probiotics in Synbiotic Fermented Milks during shelf-life

Probiotics, *Streptoccocus thermophilus* and *Lactobacillus bulgaricus* are monitoring during forty days of fermented milks *shelf-life* with the aim to observe possible correlations between prebiotic and probiotic activity. Count determination are performed on the three samples ("FY", "FYP", "SFYP") regarding *Streptoccocus thermophilus* and *Lactobacillus bulgaricus* and on "SFYP" and "FYP" regarding *Bifidobacterium lactis* and *Lactobacillus acidophilus,* with the aim to verify if eventual changes on the numbers of cfu was to attribute at the presence of prebiotics.

Regarding *Lactobacllus. delbrueckii subsp. bulgaricus* (Fig.5), cells decreased in all fermented milk samples after 40 days of storage, mainly in the first ten days. Regarding *S.thermophilus,* (Fig. 6) the number of cfu Log increased slightly in the first ten days , confirming which observed Alkalin et al., 2007: the supplementation of 1.5% whey protein concentrate to a reduced-fat fermented milk with FOS could increase the viable counts of *S. thermophilus* **.**

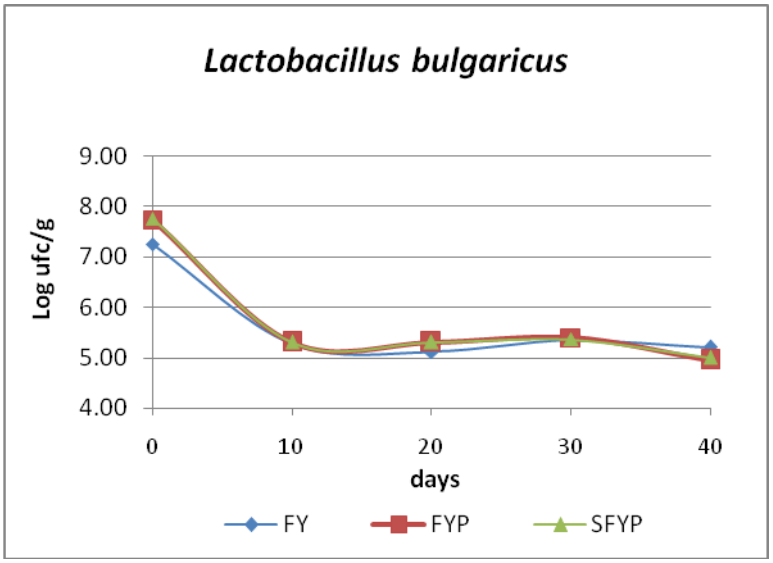

Figure 5-Analysis of *Lactobacillus bulgaricus*

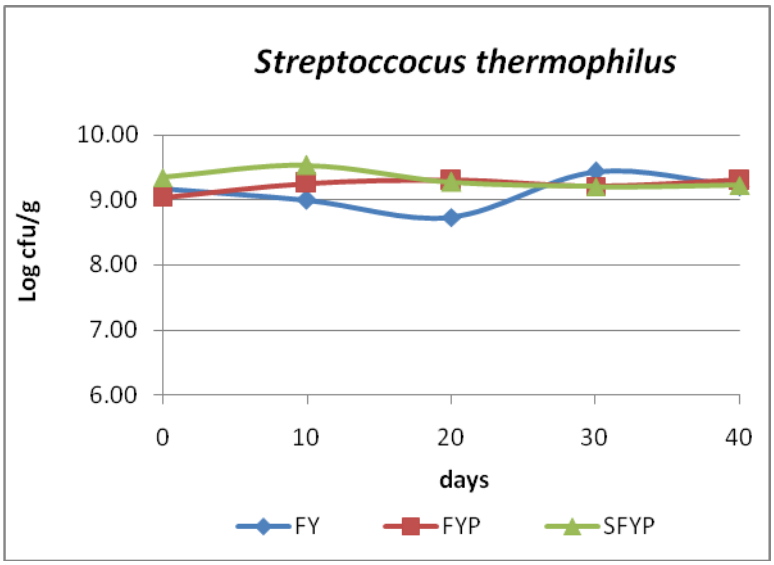

Figure 6-Analysis of *Streptoccocus thermophilus*

Regarding probiotic microorganism, 1,50X10^6 CFU/mL of *Lactobacillus acidophilus* (Fig 7*)* and 1,2 10^7 *of Bifidobacterium spp.* (Fig.8) were counted at time 0, corresponding to the time of fermented milks production , whereas after 40 days of *shelf-life, Lactobacillus acidophilus* decreased to 3.00 x10^5 while *Bifidobacterium* slightly increased to 4.10 x10^7.

The decrease of of *L. acidophilus* cfu during storage time was observed also in the control sample SFYP, where FOS and IOS are not present and probably is due to the presence of *Lactobacillus delbrueckip. Bulgaricus*, that could act as inhibitor for its growth (Dace and Shah, 1997).

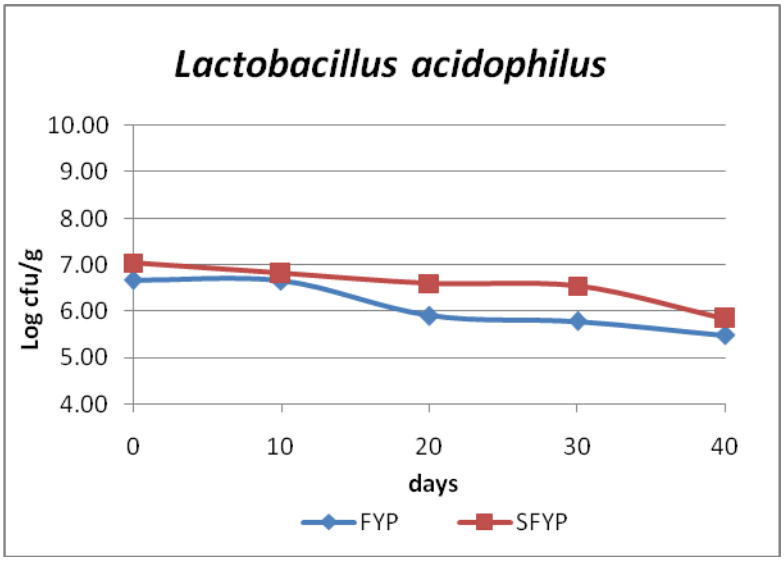

Figure 7-Analysis of *Lactobacillus acidophilus*

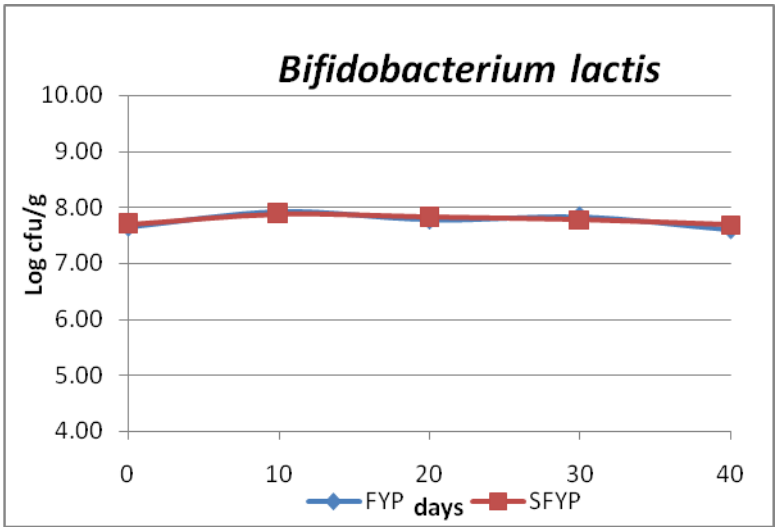

Figure 8-Analysis of *Lactobacillus acidophilus*

The pH was measured in all the samples, FY; SFYP and FYP during the forty days: values were in a range between 4.20 and 4.28, with no significative changes during samples *shelf-life*. Furthermore no variations are observed in samples with FOS and IOS according to Zhu, 2004 and Dello Staffolo et al., 2004.

In conclusion, the decrease of fructooligosaccharides and inulooligosaccharides in synbiotic fermented milks is probably due to the fact that *Bifidobacteria* microorganisms can use fructooligosaccharides owing to the production of intracellular *β-fructofuranosidases* capable of hydrolysing β(2-1) linkages (Muramatsu et al, 1992, Sangeetha et al, 2005).

7.4 Acknowledgement

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8. DISCRIMINATION OF ONIONS OF DIFFERENT COLOUR SKIN, CULTIVAR AND GEOGRAPHICAL ORIGIN BY FRUCTANS CHROMATOGRAPHIC PROFILES

8.1 Introduction

8.1.1. Chemical composition of onions

Onion nutritional composition is very complex: it contains protein, sugars, cellulose, minerals (sodium, iron), vitamins (A, C, an essential oil) and over 80 per cent water. The amount of essential oil is very small but it contains the aromatic and tear-producing properties associated with onion. These are caused by sulphides which are produced by the reaction of the enzyme alliinase on an amino acid. These substances are normally in separate cells in the tissues, but when the onion is cut and bruised, rupturing the cells, the reaction takes place. Cooking has the opposite effect, preventing the enzymatic action and thus milder and less pungent flavours are produced. (Bandyopathyay and Tewari, 1975). The balance between level of pungency and level of sugars determines the perception of sweetness in onion. High pungency can mask a high levels of sugars so that the onion is not perceived as sweet (Vågen and Slimestad, 2008).

Onion is also one of the major sources of dietary flavonoids in many countries. Onion has been characterized for its flavonol quercetin and quercetin derivates. Moreover, it is rich in other bioactive compounds such as frutooligosaccharides and sulfur compounds.

White, yellow and red onions contain a large amount of flavonoids and this content is higher in pigmented onions (Mogren and Gertsson, 2006). Onions are good sources of dietary phytochemicals with proven antioxidant properties and ability to modulate the detoxification systems : several studies have reported their functional health benefits in the reduction of cardiovascular disease risk by lowering serum cholesterol and blood pressure. They have anticarcinogenic (scavengers of free radicals), antidiabetic, anti-platelet aggregation and anti-biotic effects, they growth inhibition of tumors and microbial cells (Mudathir et al, 2008). Recently Dini et al, 2008, have studied the chemical composition of Tropeana red seeds. They have found that the protein level in Tropea red onion seeds was 24.8% and the total lipid content was found to be 20.4%, the total sugar content was found to be

21.9% and the amount of fibre was 22.4%. Regarding free amino acids, these seeds revealed large amounts of glutamic acid (97.3 mg/100 g), arginine (88.9 mg/100 g) along with lesser amounts of tyrosine (69.3 mg/100 g) and asparagine (52.3 mg/100 g). Six anions were positively identified in Tropea red onion seed. These included Cl^- , F^-, NO_3^- , PO_4^{3-} , SO_4^{2-}, and $C_2O_2^-$.

Onions can be classified according to the skin colour in yellow, white and red or in sweet and non-sweet (Vågen and Slimestad, 2008). Another classification regards short-day onions and long-day onions. Varieties listed as short-day onions bulb up when the day length is between 12 and 14 hours. Long-day onions, on the other hand, begin to form a bulb when the day length is between 14 and 16 hours.

8.1.2 Fructans distribution in onions

About 80% of onions bulb *(Allium cepa)* dry matter consists of non-structural carbohydrates (Darbyshire, 1978). The predominant of these non-structural carbohydrates are glucose, fructose, sucrose and low molecular weight fructans, whereas starch and raffinose are absent (Benkeblia et al, 2002).

In the fructans DP level is mostly between 3 and 15. Onion contains fructans with DP 3–15 of the inulin neo-series, in which β(1–2)-linked fructose chains can be attached to the fructose C1 or the glucose C6 of the sucrose starter unit (Darbyshire and Henry, 1978; Ernst, 1998; Vijin et al, 1997).Comparative chromatographic analyses of bulb fructo-oligosaccharides from sweet, storage and dehydrator-type cultivars have shown that C6-linked (neokestose) derivatives predominate over C1-linked (1-kestose) types and that cultivar differences are primarily quantitative (Shiomi et al. 1997). Onion fructans are primarily of the trisaccharides to pentasaccharide size class, but variations to size profiles occur in relation to high, medium, or low dry matter content.(Darbyshire and Henry, 1981, Suzuki and Cutcliffe, 1989, Jaime et al, 2001).

Regarding factors that could influence carbohydrates onion composition, it was observed that genetic factor is very determinant, mainly on the glucose content; instead fructose is more affected by environment. Sucrose and 1-kestose (DP3 fructans) have a transient role in the fructan metabolism (Kahane R, 2001).

The presence of reducing sugars (i.e. fructose and glucose) in onions bulb can have major effects on the quality of the processed product, since non-enzymatic browning in dehydrated products has been attributed to the reducing sugar–amino nitrogen Maillard reaction (Hodge, 1953). Short chain fructans, with a DP less than 5, are potentially used as natural low-calorie sweeteners (Smeekens, 1998) .Onion bulbs with fructans of a high DP may be used for lipid replacement with consequential health benefits (Van Loo et al, 1995).High bulb dry matter and high soluble solids are generally linked to a greater accumulation of fructans rather than mono-and disaccharides (O'Donogue et al, 2004).

The fructose, glucose, sucrose and short chain fructans are formed in the leaves. Sucrose is transported against a concentration gradient from the leaf blade via the pseudo stem to the leaf base. Kahane et al, 2001, hypothesized that this sucrose transport can be achieved by fructans polymerization in the bulb which reduces the osmotic activity considerably compared with simple NCS like fructose, glucose and sucrose. Fructan biosynthesis is initiated by the enzyme sucrose-sucrose 1-fructosyltransferase (SS1FT), which catalyses the formation of 1-kestose from sucrose (Vijin et al, 1998) .

Fructans are the main reserve of onions; oppositely to starch behaviour, they can act as osmoregulators owing to their solubility in the vacuole water (Darbyshire and Henry, 1979). There are ongoing discussions on whether fructooligosaccharides partecipate in hydric stress caused by drought or cold.

In onion FOS accumulate during bulbing and they are catabolized during the regrowth and the sprout development of the bulbs (Darbyshire, 1978).

8.1.3.PGI onions

The area cultivated with onions in Italy (in 2004) was 14.073 hectares with a yield of 304 quintals per hectare, for a total production of 423.763 tonnes.

In Italy a little of onion cultivars or geographical area are protected by Protected Designation of Origin (PDO) or by Protected Geographic Identity (PGI) like PDO "Cipollotto Nocerino" of Campania, PDO "Medicina" onions of Emilia-Romagna and, probably the most famous one, "Cipolla rossa di Tropea" .

The Protected geographical indication is the name of an area, a specific place or, in exceptional cases, the name of a country, used as a description of an agricultural product or a foodstuff

- which comes from such an area, place or country,
- which has a specific quality, goodwill or other characteristic property, attributable to its geographical origin,
- whose production, processing or preparation takes place within the determined geographical area.

In Italy onions with PGI are *Allium cepa var tropeana*, regulated by Reg CE 284 27/03/2008 and Medicina onions.

The PGI regulamentation of *Allium cepa var. tropeana* include name (4.1), the ecotypes that have a characteristic shape and are produced early owing to the effect of photoperiod: "Tondo Piatta", that it is classified as an early crop, "Mezza campana" a mid to early crop, "Allungata" a late crop. Type of products (4.2) labeled with PGI that are "Cipollotto"; "Cipolla da consumo fresco", "Cipolla da serbo"; geographical area (4.3) that are Province of Cosenza, Province of Catanzaro and Province of Vibo Valentia, proof of origin (4.4), method of production (4.5), link (4.6), inspection body (4.7), labeling (4.8).

Medicina onions labelled with PGI are early crop onions Dorata di Bologna, Densidor and Vaquero. Ecotype: Densidor; white onions: Blanco Duro, Alabaster, Nevada, Cristal; red onions Sanguigna di Milano sel. Reddy, Rossa d'Inverno sel. Rojo Duro and late crop Katty, Early Yellow Globe, Top Spring, Saratoga. Regarding fertilization nitrogen, phosphorus and potassium maximum amounts are regulated: nitrogen is distributed four times from sowing to swollen bulbs with a maximum of 130 Kg/ha for each production cycle. Phosphorus and potassium are distributed in pre-sowing phase for a maximum of respectively 50kg/ha and 300kg/ha for each production cycle.

Onion with DOP is "Cipollotto Nocerino" of Campania regulated by Reg.Ce 656 10/07/2008.

Others Italian onions have Slow food production disciplinary like the onion of Giarratana (RG, Sicilia), Red onion of Acquaviva (BA, Puglia), Red onion of Certaldo (FI, Toscana), Cipolline d'Ivrea (TO, Piemonte).

Furthermore a Scalogno denomined Scalogno di Romagna had obtained PGI and it is regulated by Reg. CE 2325/97.

8.1.4.Response of onions to irrigation and fertilization

Onion is sensitive to water stress and requires frequent and light irrigation to avoid water deficiency and to adequate recharge the plant root zone (Koriem et al,1994). Studies about irrigation indicate that the best yields occur when the soil irrigation is interrupted 2 weeks before harvest to prevent rotting and sprouting during storage (Martin de Santa Olalla et al, 2004). A microsprinker irrigation seem to be a good irrigation technique, mainly for semi-arid climate (Kumar et al, 2007). No indications about irrigation are reported in the PGI disciplinary.

Generally onions irrigation was made each 4-5 days until 10 days from onions crop, with a water quantity until 40mm, depending on soil weaving. For example for a loose soil the quantity is lower, in the range between 17 and 24mm.

Onion requires well drained, fertile soil having pH between 6.5 and 8.0 (Rajput and Patel, 2006). The principal nutrients that onions need are nitrogen (about 2.7 kg expressed for bulb ton), phosphorus (about 1.3 kg), potassium (2.7 kg), calcium (3.0 kg), magnesium (0.6 kg).

A linear correlation between moisture percentage and nitrate content was reported by Santamaria et al, 1999.

Nitrogen (N) is an essential plant nutrient, which is taken up by the crops throughout the growing season. Most common forms of nitrogen found in the soils are organic N, ammonium (NH_4^+), nitrate (NO_3^-), and gaseous nitrogen (NH_3, N_2).

Mineralization and nitrification processes convert the organic N and NH_4^+ into NH_4^+ and NO_3^- respectively which are absorbed and utilized by crops and termed as available nitrogen. Nitrate is highly mobile and leach able. Nitrate leaching potential depends on soil properties, crops and crop rotation, irrigation methods, management practices and climatic parameters (Ajdary et al, 2007) .

The amount of nitrogen fertilizer recommended for onions varies widely.

Onion responds well to additional fertilizer applied 40–60 days after transplanting. With a plentiful N supply, NO_3^- absorption by plants may exceed its reduction and assimilation within the plant.

Nitrogen has also an adverse effect on storability of onions: the crop grown with higher doses of N tend to rot and sprout earlier during storage (Kumar et al, 2007). To prevent excess absorption of NO_3^- by

plants, it has been proposed to substitute a small part of NO_3^- supply by NH_4^+ , urea, or a mixed amino acid source (Güneş et al, 1996) .

8.1.5. State of the art

Analyses on carbohydrates on onions reported in literature are performed by gel permeation chromatography technique (Darbyshire and Henry, 1978) , HPLC with refractive index (Dini et al, 2008), evaporative light scattering (Kahane, 2001, Vågen, 2008) and PAD detector (Benkeblia, 2004). Furthermore DP onions was determinate by MALDI-MS analyses by Wang et al, 1999.

Total fructooligosaccharides content was determined by AOAC enzymatical analyses (Andersen, Sørensen, 1999).

Fructooligosaccharides total content seems to be affected by storage (Benkeblia et al, 2004 and 2005): fructans content in onion bulbs tends to decrease during refrigerated, ambient atmosphere (Ernst et al, 1998) and low-oxygen storage (Ernst et al, 2003). In others works a positive correlation between fructan content and percent marketable bulbs stored at refrigerated conditions for 3-4 month was observed (Rutherford et al, 1982, Suzuki et al, 1989).

Differences on fructans cultivars are observed and reported about glucose, fructose, sucrose and total fructans content (Vågen et al, 2008) .

Recently Galdón et al, 2009, showed a discrimination between six onion cultivars from Tenerife performing a Linear Discriminant Analysis (LDA) on these following parameters: moisture, ash, proteins, total soluble and insoluble fiber ratio, glucose, fructose, total sugars and total fructans content but regarding fructooligosaccharides distribution no chemometric works are reported.

About carbohydrates content of onions with PGI label, Dini et al 2008, investigated the chemical composition of *Allium cepa L.Var.Tropeana*, analyzing fructose and raffinose as carbohydrates.

8.1.6 Aims of this work

The aim of this work was to characterize onions (*Allium cepa*) and shallots of different cultivars and from various geographical origins. White, red and yellow onions , purchased both from local markets and from Italian farmers, were analyzed by studying fructans distribution.

Particular attention was placed on the PGI onions like Medicina onions and Tropea onions to verify differences from the others one.

Many parameters like colour, caliber, bulb maturation stages, storage influence are investigated with the aim to verify if they affected chromatographic profiles.

Finally chemometric analyses are performed with the aim to differentiate them by cultivars or geographical region.

8.2.Materials and methods

8.2.1 HPAEC-PAD analyses

Elution of carbohydrates was performed at room temperature employing two columns: a Dionex Carbopac PA 1 Column (4x250mm), equipped with pre-column Carbopac PA1 (4x50mm) and a Carbopac ™ PA100 (4X250mm) with the pre-column Carbopac ™ PA100.

Standards are glucose, fructose and sucrose (Sigma), 1-Kestose (DP3), Nystose (DP4) and Fructofuranosyl-nystose (DP5) (Niko, Germany), Beneo P95™ (Orafti).

8.2.2 Samples Treatment

Allium cepa and *Allium ascalonicum* samples analyzed are reported in Table 1.

Onions are classified by colour of skin, geographical origin and caliber: of each caliber (small or big) three onions are cropped from the same ground and analyzed. Our attention is focused on *Allium cepa var. Tropeana*, which type of samples analyzed are reported in Table 2.

All samples were treated with liquid nitrogen, grinded with Moulinex and stored at -18°C.

Ten grams of onions were weighed and oven-dried at 65° C for 72 h, as previously reported by Kahane et al, 2001. Carbohydrates extraction was then performed testing three solvents, as previously reported in literature by Davis et al, 2007*:* water, aqueous methanol 62.5 % (w/w) an and ethanol (80% w/w). 0.25g of onions dried samples were accurately weighed and 25mL of the solvent was added; solution was homogenized with Ultra Turrax and placed at 80° C for 60 minutes under stirring and successively centrifuged at 8000 rpm for 30 minutes at 4° C.

Samples were then diluted 1:5 with HPLC deionized waters and filtered prior to the analysis with syringe nylon filters of 0.22 μm.

Table 1. Onions samples analyzed

***Allium cepa* samples**	**Caliber**	**Geographical origin**	**Crop**
Yellow skin			
BORETTANA	35-50	Reggio Emilia (Emilia-Romagna)	July 2008/2009
DENSITY	40-50; 60-80	Medicina (Emilia-Romagna)	August 2008/2009
SALSIERA	30-50	France	August 2008
GIALLA DOLCE	40-60	France	August 2008
DORATA NAPOLI	80-80	Campania	June 2008
DORATA BIOLOGICA	40-60	Emilia-Romagna	July 2008
EARLY YELLOW	40-50 ; 60-80	Medicina (Emilia-Romagna)	July 2009
IBRIDO AMERICANO 236IEFU	40-50 ; 60-80	Medicina (Emilia-Romagna)	July 2009
Red skin			
ROSSA DA INVERNO sel.ROJO DURO	40-50 ; 60-80	Medicina (Emilia-Romagna)	August 2008/2009
RED MECH	60-80	Emilia-Romagna	August 2008
BIOLOGICA ROMAGNA	60-80	Emilia-Romagna	July 2008
TROPEANA	40-50 ; 60-80	Monteporo (Vibo Valentia, Calabria)	July 2009
TROPEANA	40-50 ; 60-80	Campora San Giovanni (Cosenza, Calabria)	June 2009
CIPOLLOTTI TROPEA	40-50	Campora San Giovanni (Cosenza, Calabria)	Avril 2009
CALABRIA	40-50 ; 60-80	Calabria	July 2009

ALLUNGATA			
CALABRIA TROTTOLA	40-50; 60-80	Calabria	July 2009
RED MOON	40-50 ; 60-80	Medicina (Emilia-Romagna)	July 2009
White skin			
COMETA	60-80	Emilia-Romagna	August 2008
CASPER	50-70	Emilia-Romagna	August 2008
BLANCO DURO	40-50 ; 60-80	Medicina (Emilia-Romagna)	July 2009
CIPOLLOTTI PARMA	40-50	Parma (Emilia-Romagna)	July 2009
BIANCHE DI SALERNO	60-80	Salerno (Campania)	July 2009
BIANCHE CALABRIA	40-50 ; 60-80	Calabria	July 2009
Shallot			
SCALOGNO DI ROMAGNA	35-40	Emilia-Romagna	July 2008
SCALOGNO ITALIANO	40-60	Italy	July 2008
SCALOGNO FRANCESE	30-50	France	July 2008

Table 2. *Allium cepa var. Tropeana* samples

***Allium cepa var* Tropeana samples**	**Geographical origin**	**Season productivity**	**Crop date**
"Cipollotto"	Campora	September 2008-Avril 2009	2-04-2009
"Cipollotto"	Campora	December 2008-March 2009	18-02-2009
"Cipollotto"	Campora	December 2008-March 2009	11-03-2009
"Cipollotto"	Campora	January 2008-Avril 2009	17-04-2009
"Cipolla da serbo"	Campora	November 2008-August 2009	02-04-2009
"Cipolla da serbo"	Campora	January-May 2009	14-05-2009
"Cipolla da serbo"	Campora	February-May 2009	29-05-2009
"Cipolla da serbo"	Campora	February-May 2009	05-05-2009
"Cipolla da serbo"	Campora	February-	05-05-2009

		June 2009	
"Cipolla da serbo"	Campora	February-June 2009	14-05-2009
"Cipolla da serbo"	Campora	February-June 2009	29-05-2009
"Cipolla da serbo"	Campora	February-June 2009	11-06-2009
"Cipolla da serbo"	Monte Poro		

8.2.3 *Chemometric analyses*

Principal Component Analysis (PCA) was performed by SPSS package v.16.0.Discriminant analysis (DA) was performed on STATGRAPHIC Centurion Version XV (U.S)

A correlation matrix (Pearson's correlation coefficient) was calculated for all variables tested.

8.3.Results and discussion

8.3.1 Method optimization and validation

Fructans analyses were conducted by Carbopac PA1 column and pre-column, with a flow rate of 1 mL/min. A linear gradient was performed with an increasing concentration of sodium acetate from 0 to 125mM in 150 minutes, followed by column washing and initial conditioning.

The choice of this program had permitted the separation of about 50 peaks, both simple sugars and oligosaccharides. Glucose, fructose, sucrose, 1-kestose, nystose, fructofuranosyl-nystose are identified by comparing peaks retention times with commercial standards (Figure 1).

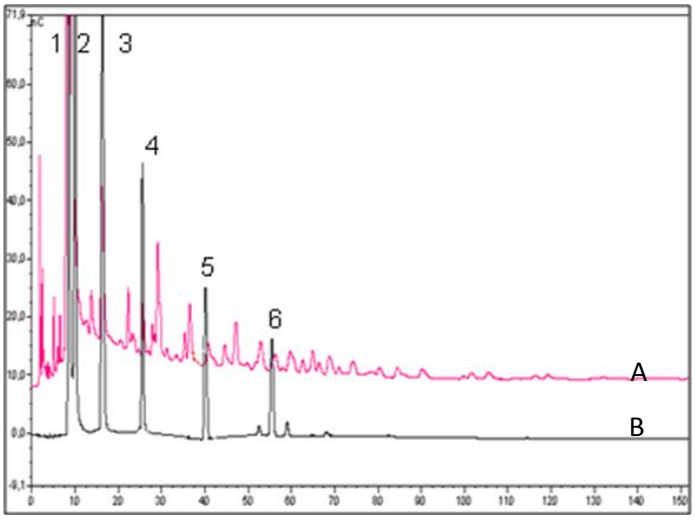

Figure 1.Chromatographic profiles A. Onion chromatographic profile; B.Standard mixture chromatographic profile 1-Glucose; 2-Fructose; 3-Sucrose; 4-1-kestose; 5-Nystose; 6-Fructofuranosyl-nystose.

Inulooligosaccharides (IOS) with DP <7 and fructooligosaccharides (FOS) with DP range between are identified by comparing Beneo P95™ (as described in the Chapter 6) and onion chromatographic profiles, as reported in Figure 2. The peaks between fructooligosaccharides and inulooligosaccharides could represent oligosaccharides with (1→6) branching.

Another method of elution was then optimized with the aim to reduce time analysis for the quantification of oligosaccharides which standard are commercially available. The column choice was a Carbopac PA100 with the pre-column Carbopac PA100, a very useful column for oligo and polisaccharides analysis.

Initially sodium hydroxide was maintained constant to 48 mM for ten minutes then it was increased to 96mM, after the first 15 minutes sodium acetate concentration was increased by gradient steps, as reported in Table 3. This method had permitted to reduce total run time for analysis from 150 to 85 minutes with a good peak resolution and a total number of peak detected about of 30 peaks, as reported in Figure 3. An internal standard, the melezitose was added to all samples with the aim to verify the repeatability of retention times and to quantify fructooligosaccharides.

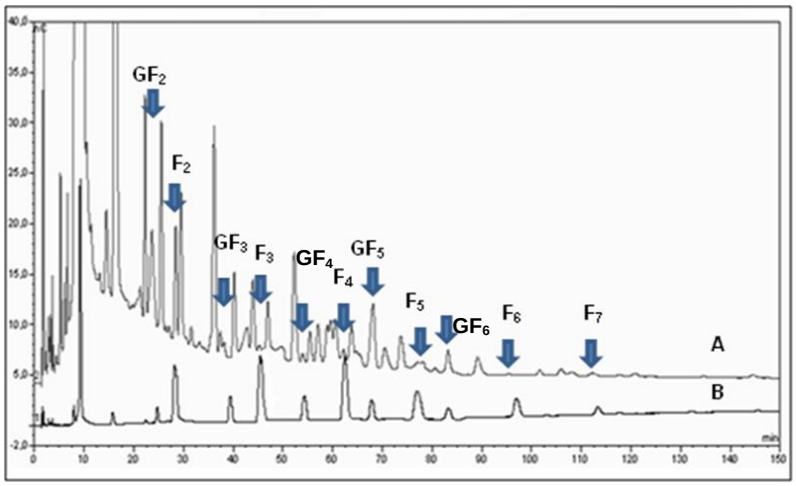

Figure 2. A-Onion chromatographic profile, B-Beneo P95™ chromatographic profile GFn: fructooligosaccharides; Fn: inulooligosaccharides; n= number of glucose molecules.

The method was validated in terms of limit of detection, limit of quantitation, intraday and interday precision and recovery on blank because all onions analyzed have oligosaccharides and therefore they could not employed as matrix.

Table 3-HPAEC-PAD method

Time(min)	A (H_20)	B (NaOH 0,6M)	C (NaAC 0,5M)
-40.0 [a]	92	8	0
0	92	8	0
10	92	8	0
15	84	16	0
40	84	16	10
50	59	16	25
60	44	16	40
85	35	25	40

[a] Negative time (column conditioning) indicates time prior analysis

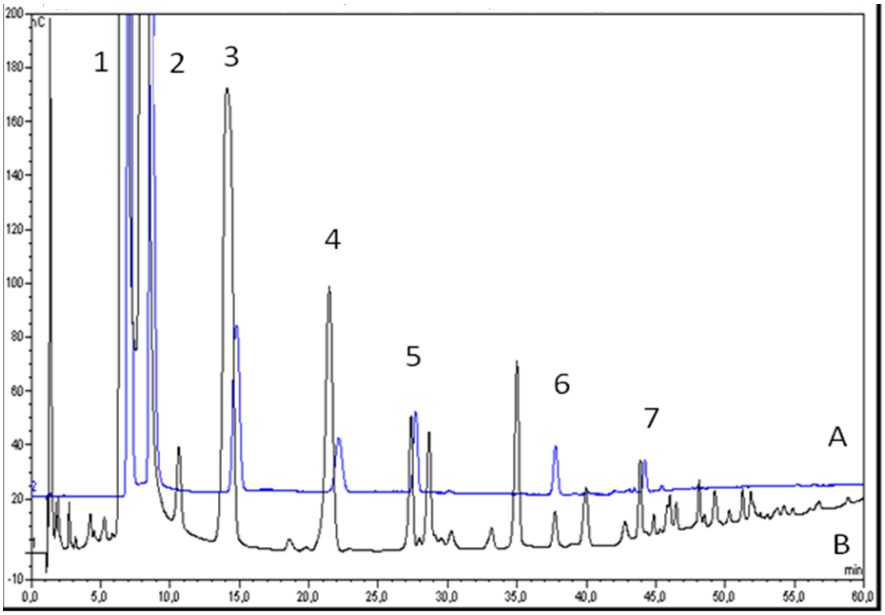

Figure 3.A-Chromatographic profile of a standard mixture B-Chromatographic profile of onion sample: 1-Glucose; 2-Fructose; 3-Sucrose; 4-Melezitose; 5-1-kestose; 6-nystose; 7-fructofuranosyl-nystose.

Limit of detection and quantitation are calculated by the following formula:

$$LOD = \frac{3\sigma_b}{b} \qquad LOQ = \frac{10 \cdot \sigma_b}{b}$$

where σ_b = standard deviation of blank; b= slope of each standard curve and results about LOD and LOQ values are reported in Table 4.

Table 4-LOD and LOQ values (µg/mL)

	Glucose	**Fructose**	**Sucrose**	**1-Kestose**	**Nystose**	**Fructofuranosyl-nystose**
LOD	0.01	0.02	0.08	0.01	0.05	0.06
LOQ	0.03	0.07	0.27	0.05	0.17	0.17

Intraday precision and repeatability were calculated both on peak area and on retention times as coefficient of variation (CV%) injecting independent standard mixture respectively 8 times in one day and 16 times in two non consecutively days.

Good results are resulted both in terms of area precision (CV%: 1.88÷6.50) and retention time (CV%:0.63÷5.77), as reported in Table 5.

Table 5- Inter-day and intra-day precision values

Area	**Glucose (200ppm)**	**Fructose (200ppm)**	**Sucrose (206ppm)**	**1-Kestose (102 ppm)**	**Nystose (102ppm)**	**Fructofuranosyl-nystose (50.4ppm)**
Intraday precision(n=8)	1.88%	2.00%	1.90%	3.68%	3.98%	2.87%
Interday precision (n=16)	4.22%	5.98%	5.77%	5.67%	4.89%	6.50%
RetentionTime	**Glucose (200ppm)**	**Fructose (200ppm)**	**Sucrose (206ppm)**	**1-Kestose (102 ppm)**	**Nystose (102ppm)**	**Fructofuranosyl-nystose (50.4ppm)**
Intraday precision(n=8)	4.22%	5.98%	5.77%	5.67%	4.89%	6.50%
Interday precision(n=16)	3.86%	4.26%	3.46%	1.86%	0.75%	0.63%

Values are expressed in terms of CV%.

Linearity was established for each standard at five equidistant levels of concentration in triplicate , verifying that curve passes through the origin with R^2>0.990 (Table 6).

Recovery was determined by spiking carbohydrates standard at three different concentration (corresponding to 10, 20 and 30%) to onion sample following this formula:

$$\text{Recovery}(\%) = \frac{C1 - C2}{C3} * 100$$

Recovery values obtained were in the range between 91.17÷102.20%, as reported in Table 7.
Where C1 = concentration determined in fortified sample;C2 = concentration determined in unfortified sample;C3 = concentration of fortification

Table 6-Linearity

Carbohydrates	b	R^2
Glucose (50-250ppm)	1.663	0.992
Fructose (50-250ppm)	1.418	0.993
Sucrose (25-125ppm)	1.024	0.994
1-Kestose (2-102ppm)	0.814	0.995
Nystose (2-102 ppm)	0.762	0.991
Fructofuranosyl-nystose (0.4-50.4 ppm)	0.558	0.998

Table 7-Values of recovery percentages

Recovery (%)	10%	20%	30%
Glucose	96.20	93.23	94.70
Fructose	93.56	91.17	93.37
Sucrose	93.68	96.05	96.64
1-Kestose	91.37	94.43	94.15
Nystose	94.30	91.35	94.04
Fructofuranosyl-nystose	94.52	92.52	102.20

8.3.2 Determination of dry matter

Dry matter was determined for all onions as described in Materials and Method. Measurement are performed in triplicate on three sample of the same cultivar for each caliber and the mean values of dry matter are reported in Table 8.

Percentages of dry matter are in the range from 6.90 to 12.15 for red skin onions (with values slightly lower for red onions from Calabria than Emilia-Romagna onions); from 6.94 to 13.73 for yellow skin onions, from 5.54 to 11.72 for white skin onions, from 13.79 to 15.70 for shallots. Shallots resulted to have a major percentage of dry matter (mean value:14.71%, n=9), followed by red skin (mean value: 9.71%, n=60) and yellow skin onions (mean value:9.70, n=36), white skins onions (mean value:8.75%, n=21) are the last.

Differences noticed are not between different colour skin onions and cultivars but also for different caliber of the same cultivars. Some standard deviation are resulted high probably because the three

onions of the same cultivar and caliber are harvested in different areas of the field, some of which more irrigated.

After thirty days of storage dry matter percentage was generally increased in all samples except than in shallot ones, in Var *Rojo duro* , in *Allium cepa var Borettana* and in *Allium cepa var Density*; the variability can probably due to the fact that the onions examined were of different age and were kept under different storage conditions.

Table 8-Dry matter (%) of onions

Red skin	**Caliber**	**Dry matter (%)** Mean value ± Sd
Rojo Duro, 2008	60-80	9.43±0.14
Red Mech, 2008	60-80	8.38±0.54
Tropea“allungata”,2008	60-80	7.77±0.18
Tropea “mezza campana”,2008	60-80	7.29±0.25
Tropea “tondo piatta”,2008	60-80	6.90±0.09
Biologica Romagna,2008	60-80	7.63±0.24
Tropea “Monte Poro”, 2009	30-40	9.65±0.93
Tropea “Monte Poro”, 2009	50-60	8.25±0.57
Red Moon,2009	50-60	11.33±1.81
Red Moon,2009	60-80	11.97±0.53
Tropea “Campora”,2009	30-40	8.18±0.41
Tropea “Campora”,2009	60-80	7.96±0.83
Rojo duro, 2009	30-40	9.68±0.43
Rojo duro, 2009	60-80	11.28±0.86
Calabria trottola, 2009	50-60	7.63±0.83

Calabria trottola, 2009	60-80	7.31±0.59
Calabria allungata, 2009	40-60	7.26±1.18
Cipollotti Tropea "Campora"	30-40	10.31±0.65
Cipollotti Parma	20-30	12.15±1.45
Cipollotti Parma	30-40	11.87±0.27
Yellow skin	**Caliber**	**Dry matter (%)** Mean value±Sd
Salsiera, 2008	30-50	13.73±0.13
Borettana, 2008	35-50	10.58±0.50
Density, 2008	60-80	10.49±0.22
Dorata biologica, 2008	40-60	6.94±0.05
Gialla dolce France, 2008	40-60	7.12±0.16
Dorata Napoli,2009	80-80	7.77±0.14
Ibrido Americano, 2009	40-60	10.13±0.70
Borettana,2009	40-50	10.04±0.86
Density,2009	40-50	8.72±0.64
Density,2009	60-80	10.99±1.32
Early Yellow ,2009	60-80	10.59±0.78
Early yellow,2009	40-60	9.27±0.54
White skin	**Caliber**	**Dry matter (%)** Mean value±Sd
Casper,2008	50-70	7.89±0.46
Cometa, 2008	60-80	5.54±0.05
Cipollotti Parma, 2009	40-50	11.09±1.35
Blanco duro,2009	60-80	11.72±1.17

Bianche calabria,2009	40-50	8.04±0.45
Bianche calabria,2009	60-80	7.75±1.03
Bianche Salerno, 2009	60-80	9.21±1.12
Shallots	**Caliber**	**Dry matter (%)** Mean value±Sd
Scalogno di Romagna,2008	35-50	14.64±0.16
Scalogno italiano, 2008	40-60	13.79±0.30
Scalogno francese, 2008	30-50	15.70±0.99

8.3.3 First consideration about differences on colour skin and cultivars and quantitative determination of FOS in onions

Preliminary analyses are conducted to onions cropped in the year 2008, to verify if carbohydrates chromatographic profiles changed between different colour skins and shallots.

As showed by some chromatographic profiles representing red onions (Figure 4), yellow onions (Figure 5) white onions (Figure 6) and shallots (Figure 7), many differences were observed from chromatographic profiles. These differences are showed mainly in the number of peaks representing oligosaccharides at higher degree of polymerization, that are most present in shallots. Red onions have similar chromatographic profiles, while a great variability was observed between yellow onions; for example "Salsiera" onions have a more similar oligosaccharides distribution to shallot samples, while "Gialla dolce" chromatographic profile is comparable to which of red onion samples. White onions have very little peaks in correspondence to oligosaccharides region.

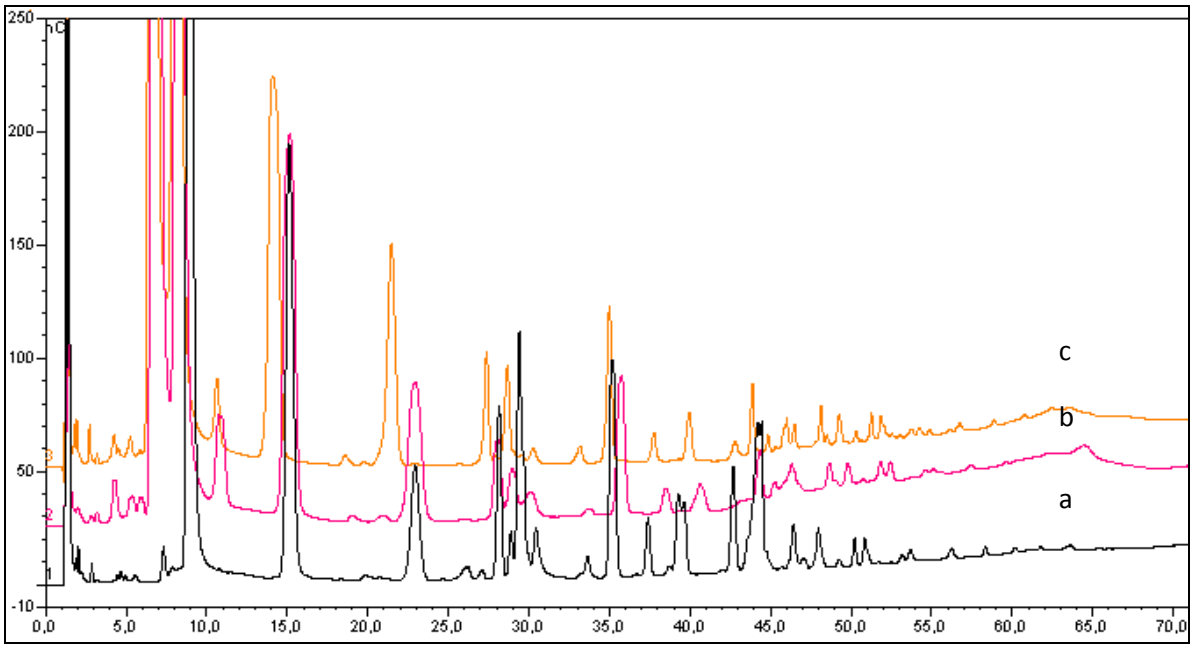

Figure 4. Red onions chromatographic profiles a- "Red Mech"; b-"Biologica Romagna", c- "Tropea allungata".

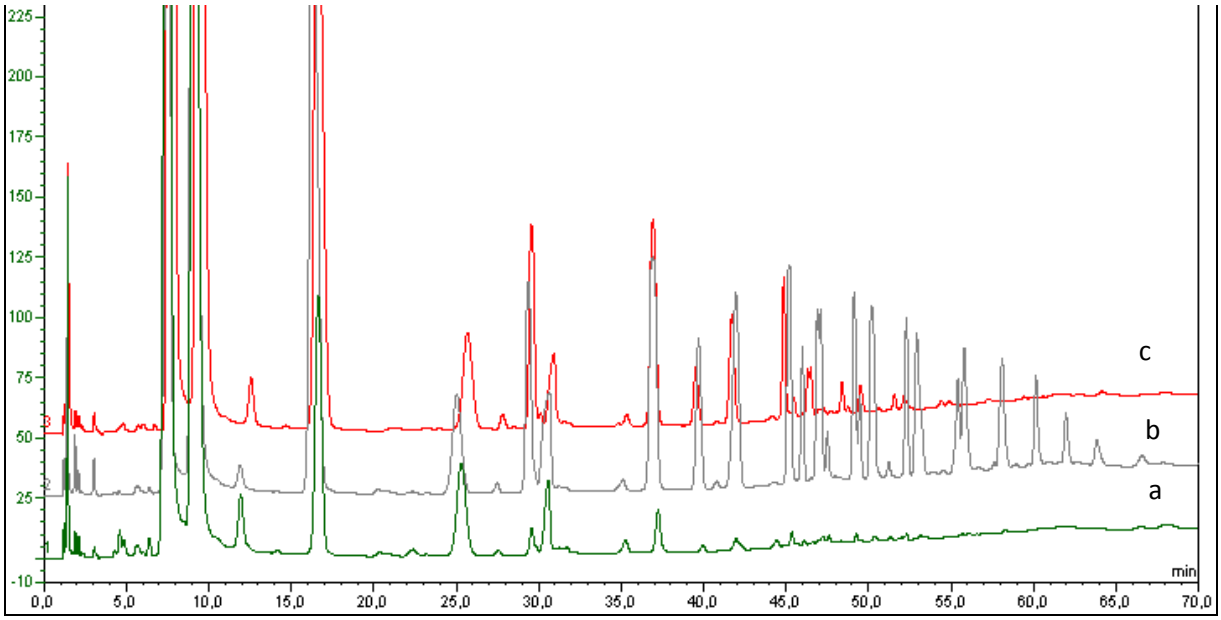

Figure 5. Yellow onions chromatographic profiles a- "Gialla dolce"; b-"Salsiera"; c-"Density"

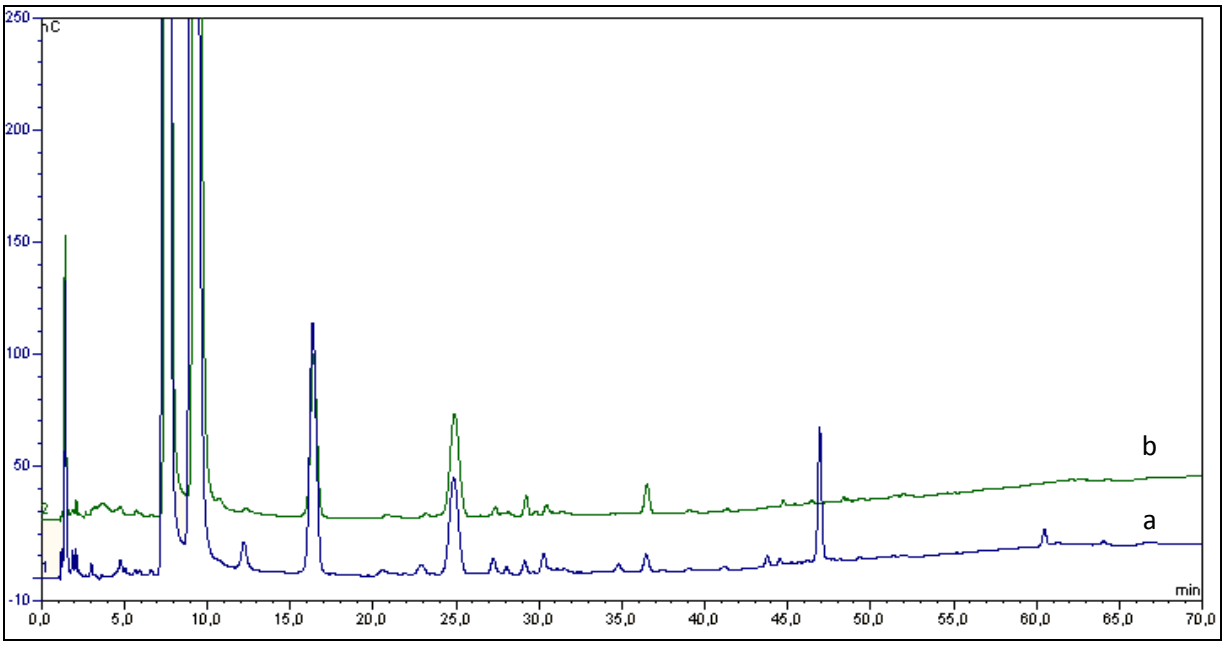

Figure 6. White onions chromatographic profiles a- "Casper"; b-"Cometa".

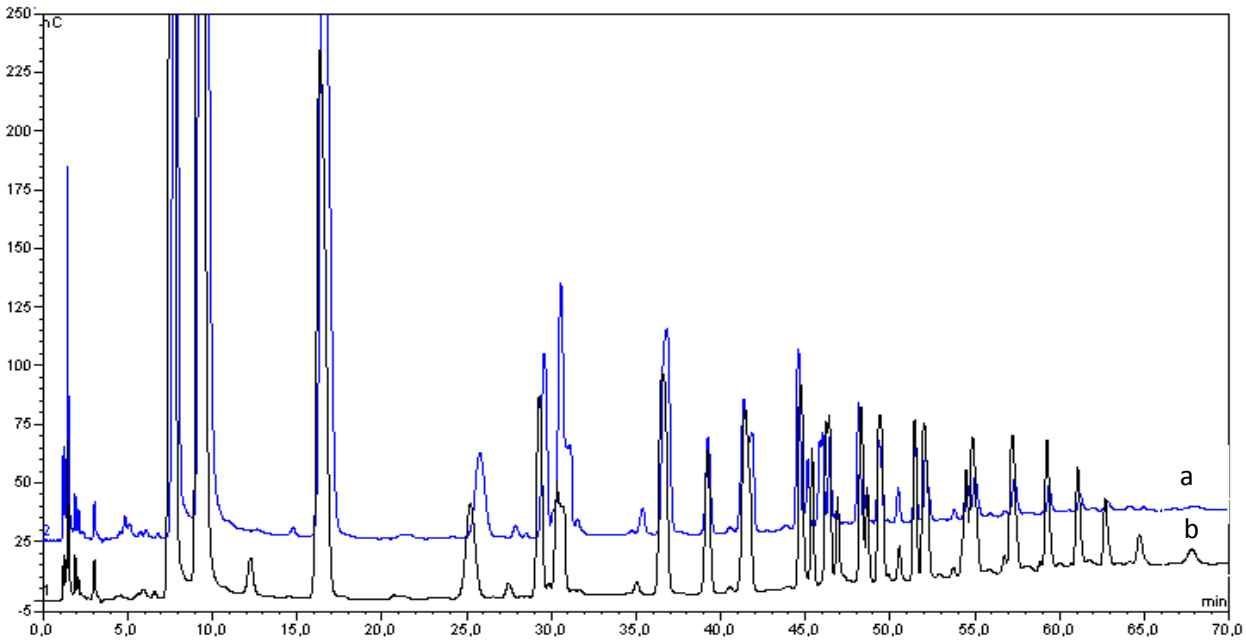

Figure 7.Shallot chromatographic profiles a-Shallot from Italy b-Shallot from France

In Figure 8 it is reported a comparison between chromatographic profiles of red, yellow, white onions and shallots and on the box is marked oligosaccharides region.

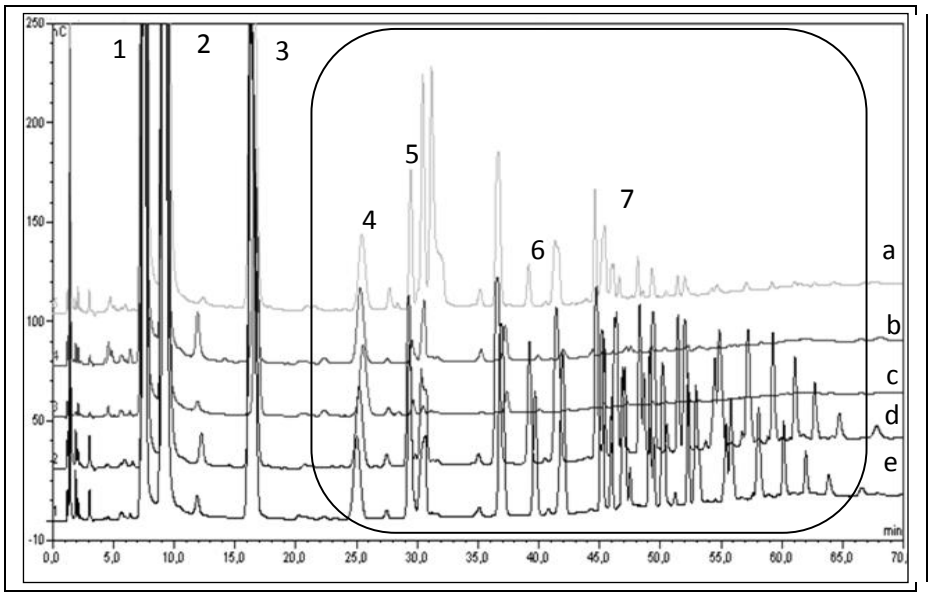

Figure 8. Comparison of some onion sample chromatographic profiles: a-Var. Red mech, b-"Gialla dolce" , c-white Var. Cometa, d-shallot, e-s"Salsiera" yellow onion; 1-Glucose; 2-Fructose; 3-Sucrose; 4-Melezitose (ISTD), 5-1-Kestose; 6-Nystose; 7-Fructofuranosylnystose

Percentage composition of non-structural carbohydrates has been found different for onions cultivars: glucose, fructose and sucrose were the main sugars in all samples, followed by tri-, tetra- and pentasaccharides but the relative ratios were variables. The greatest differences are shown between FOS kestose, nystose and fructofuranosy-nystose percentages in shallots (Figure 9-A) and yellow "Salsiera" onion (Figure 9-B), where the sum of GF2, GF3 and GF4 represent 13% of non-structural carbohydrates respect Tropea (Figure 10-A) and white onion (Figure 10-B), where the sum is lower than 1% Tropeana and white onions . In this last variety of onions the high content of simple sugars confirmed its famous "sweetness" .

Quantitative analyses were conducted on sugars and FOS 1-kestose, nystose and fructofuranosyl-nystose both on onions and shallot samples after harvested and on onions.

We observed a negative correlation between simple carbohydrates (Table 9-A) and FOS content concentration (Table 9-B). Furthermore in onion samples where fructose was much higher than sucrose concentration, like in white onions samples, FOS were in a little percentage. From these data we can hypothesize that the ratio sucrose/fructose could be an important parameter to predict fructans production; in fact, as reported by Douglas et al., 2008, sucrose may play a central role and control the ability of the plant to synthesize fructans. Analyses performed on samples stored during thirty days (Table 10 A and B) showed a decrease of FOS concentration in all onions, in agree with Jaime et al, 2001, probably due to enzymatic hydrolysis of fructans.

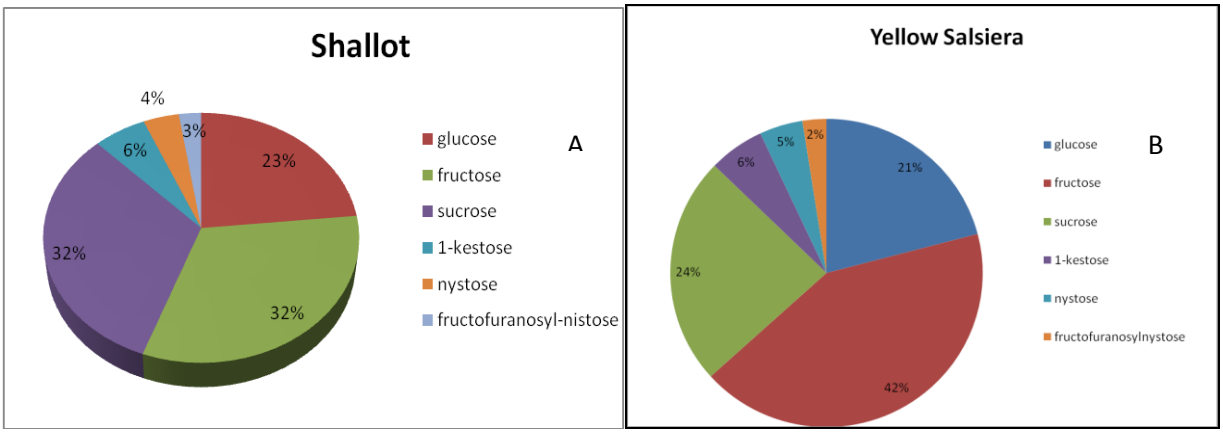

Figure 9.Percentages of carbohydrates in A:shallots sample B: yellow "Salsiera"onion.

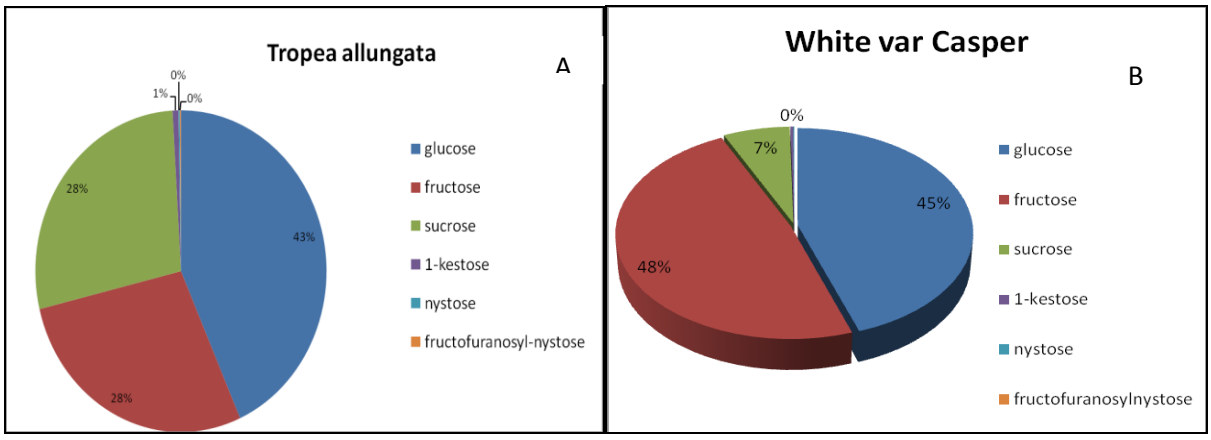

Figure 10.Percentages of carbohydrates in A: Tropea allungata red onion; B: White onion variety Casper

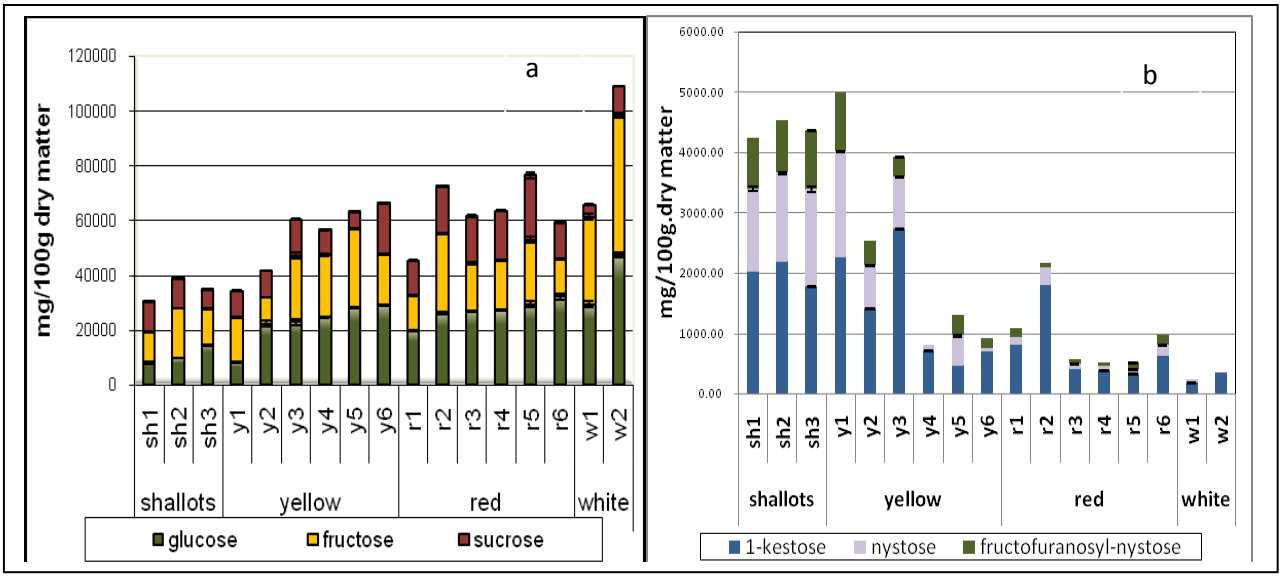

Table 9- Concentration (mg/100g DM) of a- glucose, fructose, sucrose b- Concentration (mg/100g DM) of FOS (DP3-5) in onions and shallots

Where: sh1:shallot italiano; sh2: Shallot of Romagna; sh3.Shallot of France; y1: "Salsiera"; y2: "Borettana"; y3:"Density";y4: "Dorata biologica"; y5: "Dolce Francia"; y6:"Dorata Napoli"; r1: "Rojo duro"; r2: "Red Mech"; r3: "Tropea allungata"; r4: "Tropea tondo piatta"; r5: "Tropea mezza campana"; r6: "Biologica Romagna"; w1: "Casper"; w2: "Cometa".

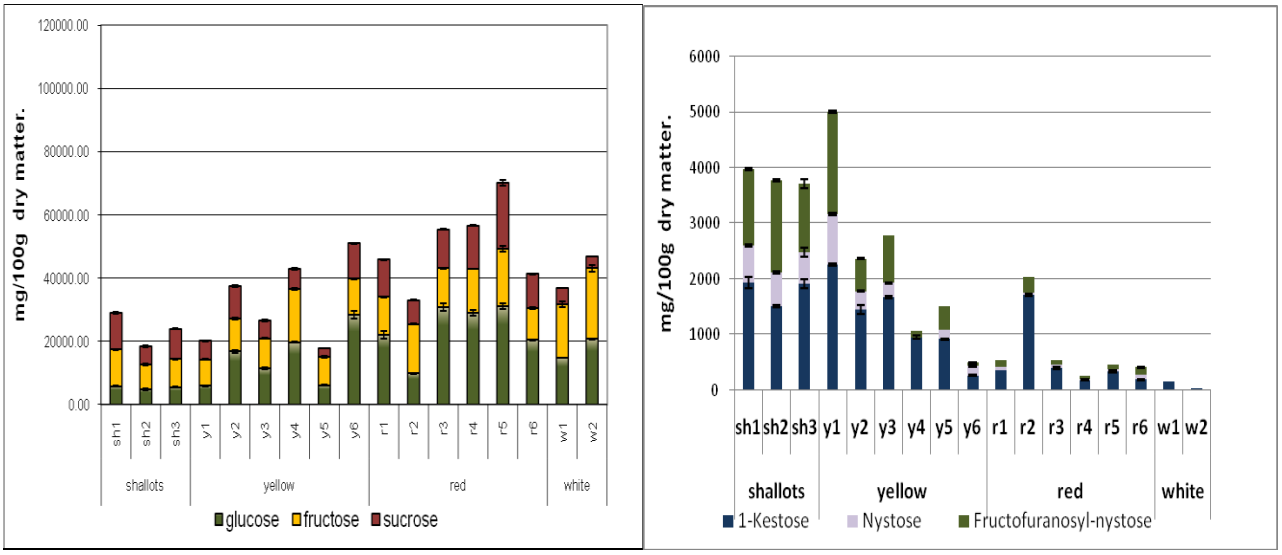

Table 10- Concentration (mg/100g DM) of a- glucose, fructose, sucrose b- Concentration (mg/100g DM) of FOS (DP3-5) in onions and shallots after 30 days of storage

Where: -sh1:shallot italiano; sh2: Shallot of Romagna; sh3.Shallot of France; y1: "Salsiera"; y2: "Borettana"; y3:"Density";y4: "Dorata biologica"; y5: "Dolce Francia"; y6:"Dorata Napoli"; r1: "Rojo duro"; r2: "Red Mech"; r3: "Tropea allungata"; r4: "Tropea tondo piatta"; r5: "Tropea mezza campana"; r6: "Biologica Romagna"; w1: "Casper"; w2: "Cometa".

8.3.4 Fructooligosaccharides distribution in Tropeana onions

Fructooligosaccharides distribution in *Allium cepa var tropeana* was studied in relationship with some parameters, like shape, caliber, ground, bulb maturation stage and storage.

Two type of onions were studied, reported in the PGI disciplinary:"Cipollotto" and "Cipolla da serbo". On green onions, analyses are conducted both on stalks and on bulbs. About stalks chromatographic profiles, only simple sugars are detected (data no shown).

8.3.5 Evaluation of carbohydrates distribution during bulb maturation

Tropeana onions are cropped from seedling to bulb maturation , on each fifteen days and subdivided in five stages. As reported in Figure 10-a, representing the seedling chromatographic profile, not fructooligosacchides are present. Fructan biosynthesis is initiated by the enzyme sucrose-sucrose 1-fructosyltransferase (SS1FT), which catalyses the formation of 1-kestose from sucrose (Vijin et al, 1998). Chain elongation is catalyzed by fructan:fructan 6G-fructosyltransferase (6G-FFT) and fructan:fructan1-fructosyltransferase (1-FFT) activities). During bulb maturation (Figure 10-b, c ,d, e), no qualitative differences about chromatographic profiles are showed.

Regarding quantitative determination, differences are observed about onions of small caliber and big caliber, cultivated and harvested in the same days. In fact, as reported in Figure 11, relative to the two last bulb onions maturation step, bigger caliber have for example an higher concentration of simple sugars

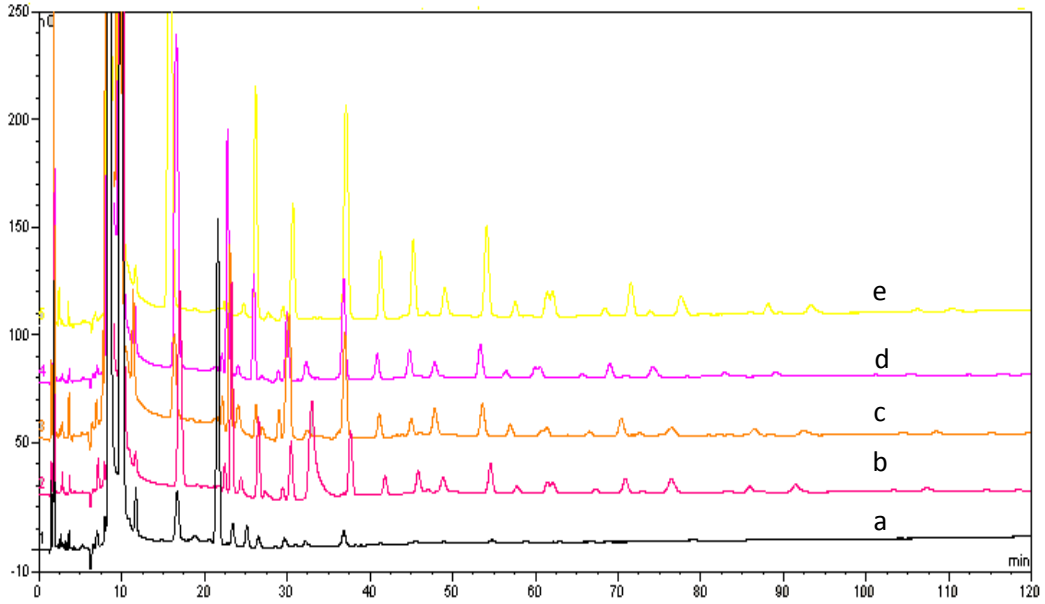

Figure 10-Chromatographic profiles : a-seedling; b-after 18 days; c-after 27days; d-after 42 days; e-after 55 days

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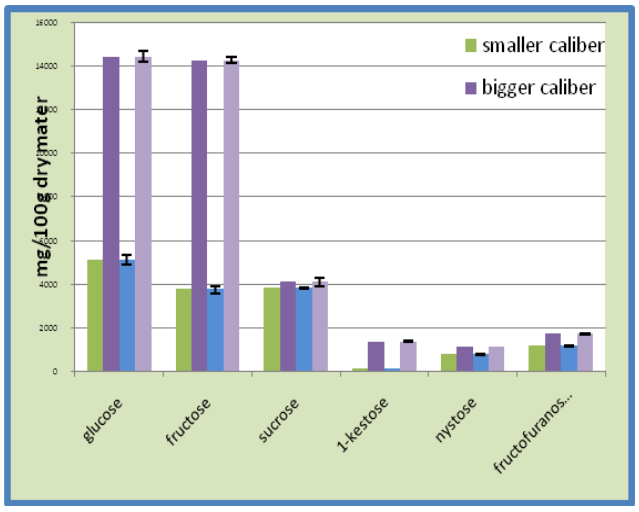

Figure 11. Comparison between small and big onions caliber regarding maturation steps.

In Figure 12 it is reported the composition of simple sugars and FOS during bulb onions maturation relative to onions of bigger calibers. It is possible to observe that all carbohydrates concentration, both simple sugars and fructooligosaccharides with DP range 3-5 initially increased (in the first 20 days)

and then decreased. Only sucrose quantity is higher after 55 days. We could consider 42 days the optimal maturation step for onions harvesting to obtain the maximum carbohydrates concentration; although nystose quantity is higher on onions harvested after 27 days of maturation.

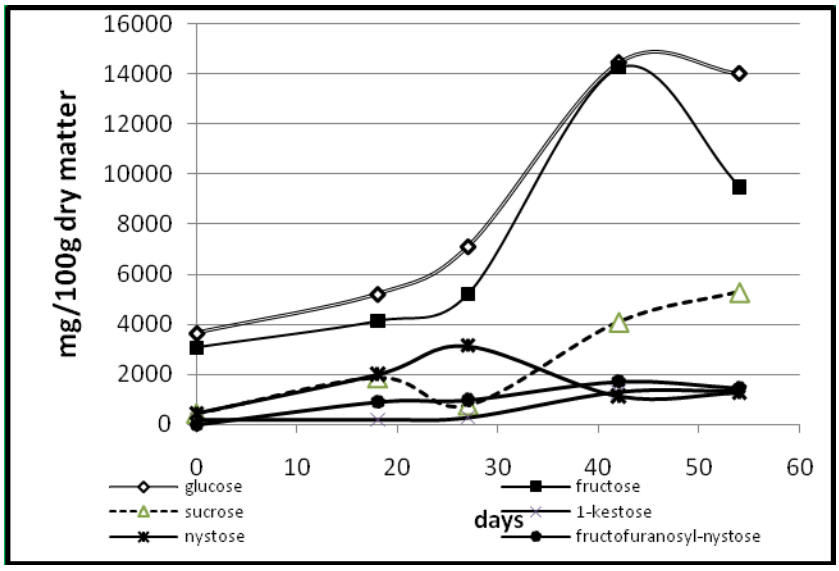

Figure 12.Carbohydrates concentration during 55 days of onions maturation.

8.3.6. Geographical Area

PGI disciplinary requires that Tropeana red onions are cultivated in geographical area of Province of Cosenza, Province of Catanzaro or Province of Vibo Valentia. Two area, with soil properties very different, are examined: one, "Campora San Giovanni", Amantea (province of Cosenza) and the other Monte Poro (province of Vibo Valentia). In Amantea area there is the principal production of this PGI red onions, though many onions that are produced in this area are then packaged and sold at Tropea.

Campora San Giovanni grounds are nearer to the sea, where the soil is principally composed of sandstone and clay. Therefore it is a soil poor of micronutrients , that it needs of a continuous irrigation and fertilization. In Campora area onions are principally harvested in June.

Monte Poro is instead collocated at an altitude of 710 m and the soil is more rich of micronutrients. In this area the onions production is later than the others area: the onions harvest begins at July.

Six onions (of two different caliber) samples of each soil are collected at the end of onion maturation and analyzed to verify if there was differences about their carbohydrates chromatographic profiles. Internal differences about onions from the same geographical area are not observed. As showed by Figure 13, where two chromatographic profiles of onions of the same caliber (60-80mm) and the same bulb maturation step, from " Campora San Giovanni" (A) and "Monteporo" (B) are reported, not qualitative differences between fructooligosaccharides distribution are observed. Quantitatively "Campora San Giovanni" showed higher peaks area correspondent to oligosaccharides eluted between 20 and 30 minutes, with a lower DP, probably due to the highest water and micronutrients provided.

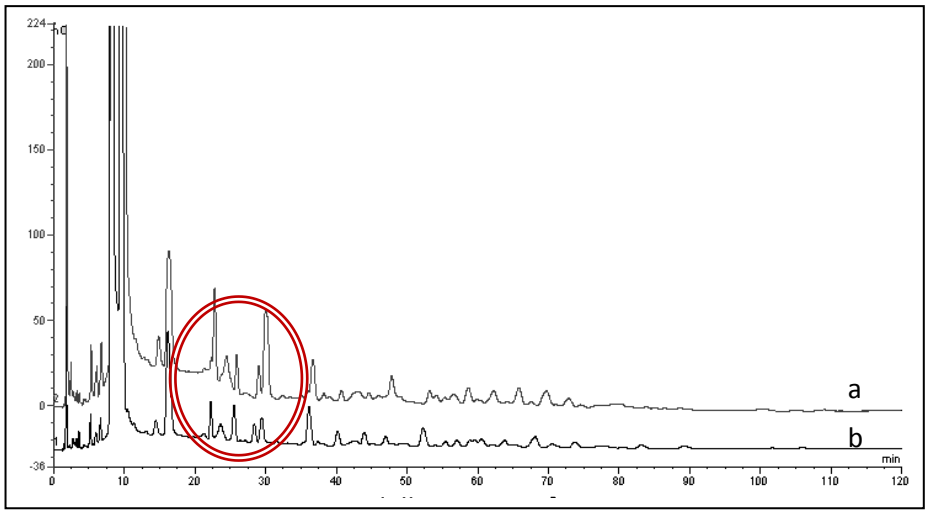

Figure 13. Chromatographic profiles of *Tropeana* onions a-cultivated in "*Campora*" area b-cultivated in *"Monteporo"* area.

8.3.7.Type of product

Two types of product reported in the PGI disciplinary were analyzed: "Cipollotto" and "Cipolla da Serbo" , cultivated on "Campora San Giovanni" area. As firstly reported for the other parameters no qualitative differences are observed , while a smaller content of simple sugars and FOS are present in "Cipollotto" samples, in accord to which observed for onion maturation steps (Figure 14).

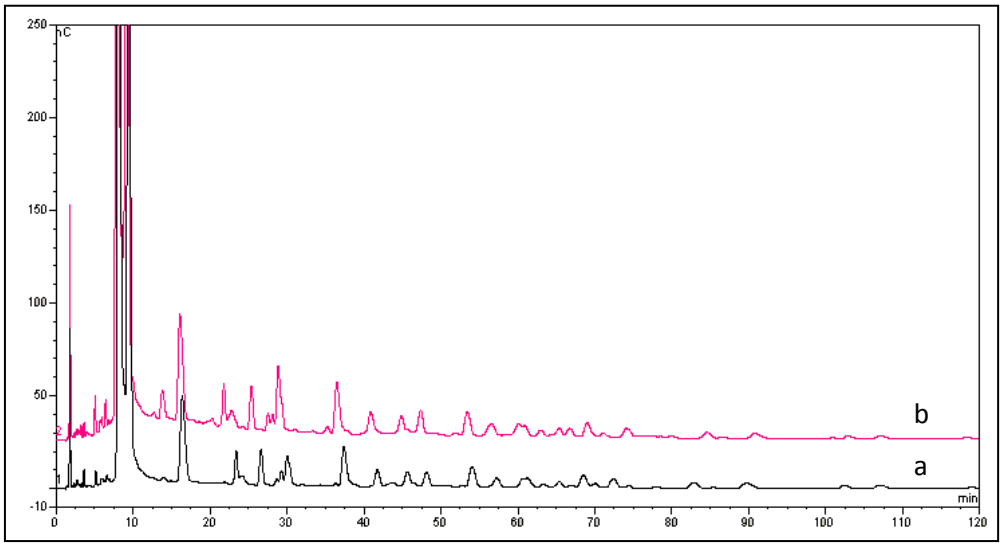

Figure 14.Chromatographic profiles of a-"Cipollotti"; b-"Cipolla da serbo"typology.

8.3.8. Correlation between carbohydrates and dry matter

Regarding red onion analyses, possible correlations between simple sugars, dry matter percentage and fructans content in onions were studied. A correlation between moisture and total fructans can be seen (r=-0.601), as previously described by Jaime et al., 2001 and Rodríguez-Galdón et al, 2009 (Figure 15). Total fructans are calculated as sum of area percentages, as previous described in the Chapter 6. The proportion of high molecular weight compounds increased as DM increased, as for example for shallots, because the polymerization of nonstructural carbohydrates reduces their osmotic activity and enables the accumulation of carbohydrates, increasing DM content, confirming the osmoregulator role attribute to

fructans (Jaime et al, 2001). According to Darbyshire and Henry, 1979, the profile of fructans distribution suggested that these compounds have been hydrolyzed to fructose in low dry weight varieties (e.g. Tropeana red onion) to facilitate osmoregulation as the bulb takes up water and expands during bulb developing, and water content is therefore higher than in high dry weight cultivar where fructans are not hydrolyzed because of their genetically controlled inability to take up water, either from their inability to hydrolyze fructans to free fructose or from the behavior of the cells of high dry weight cultivars that may have restricted water uptake.

A strong correlation between glucose and sucrose are found (r=0.843), as previously described by Rodríguez-Galdón et al, 2009. This correlation defines the following regression line that makes it possible to determinate the content of one sugar when the concentration of the other known:

[Glucose (g/100g)]=0.88 x [fructose (g/100g)] +24.37. This phenomenon has been observed for other fruits such as bananas (Forster et al, 2002) and tomatoes (Hernández et al, 2008) and suggest a common origin for both sugars, probably from the sucrose. Sucrose is the sugar used for the mobilization of carbon is vascular plants. It can be synthesized from triose phosphate produced in the photosynthesis, or from starch of the chloroplast, during the night (Li et al, 1992).

A very strong correlation was discovered between total fructans and the sum of simple sugars (glucose, fructose and sucrose), as previous found about FOS determination curing sample storage.

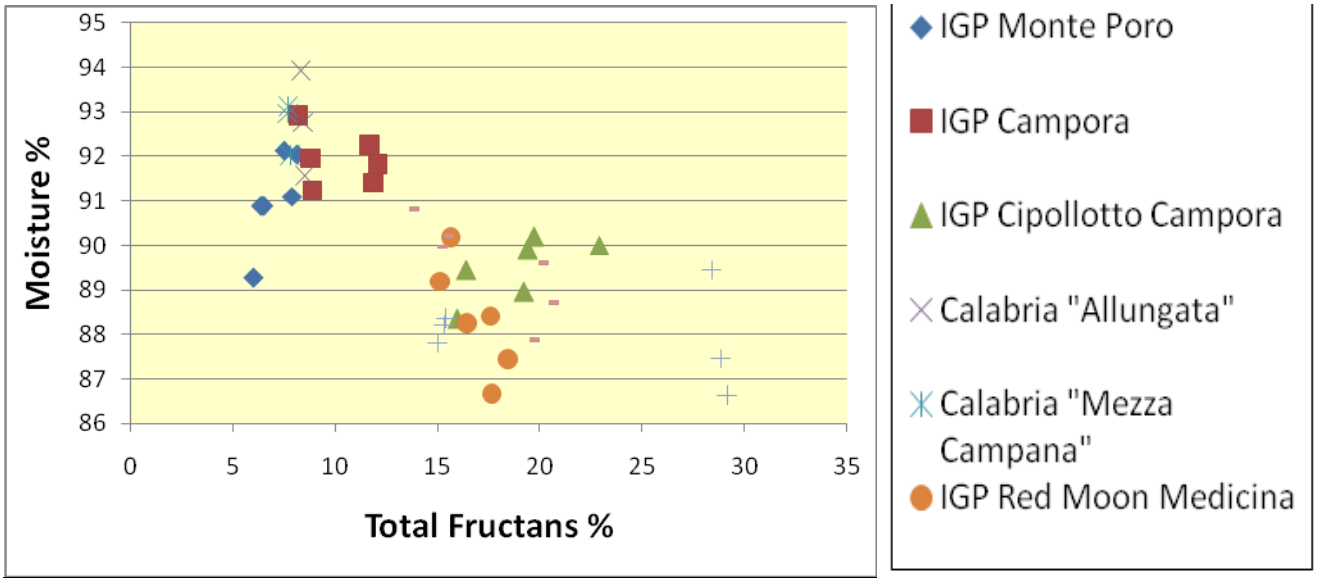

Figure 15-Correlation between total fructans and moisture (r=- 0.601; y=-0.19x+93.16)

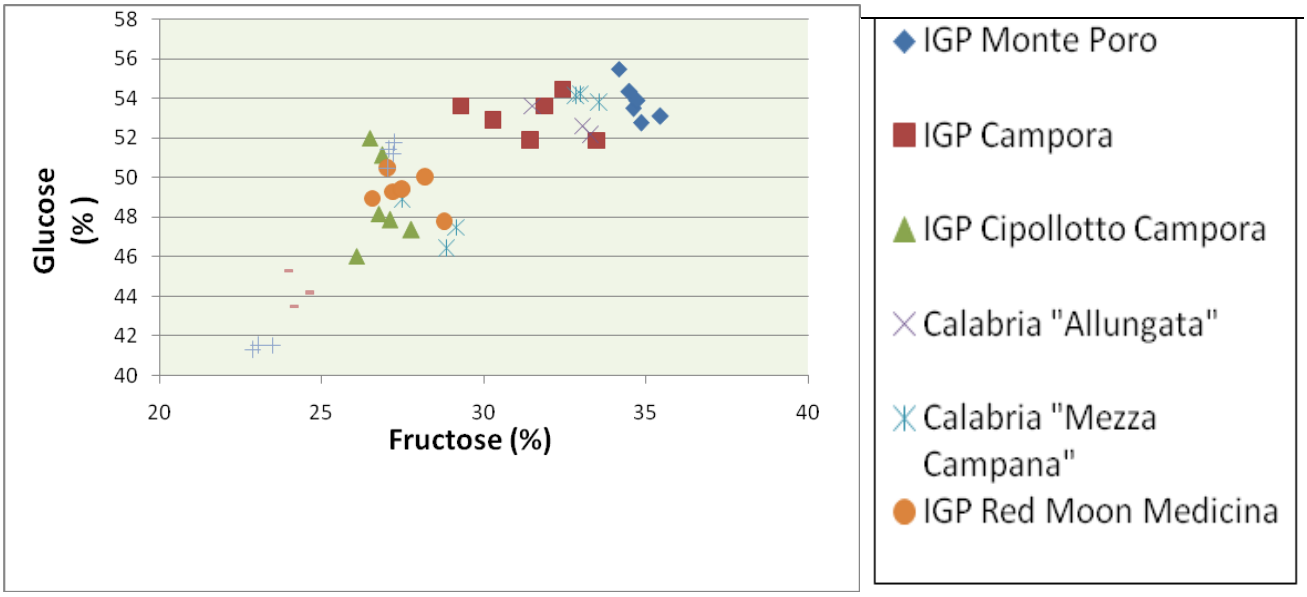

Figure 16. Correlation between glucose, calculated as (Area Glucose/Total area (Sugars+Fructans)*100) and Fructose, calculated as (Area Fructose/Total area (Sugars+Fructans)*100) (r= 0.843; y=0.88x+24.37)

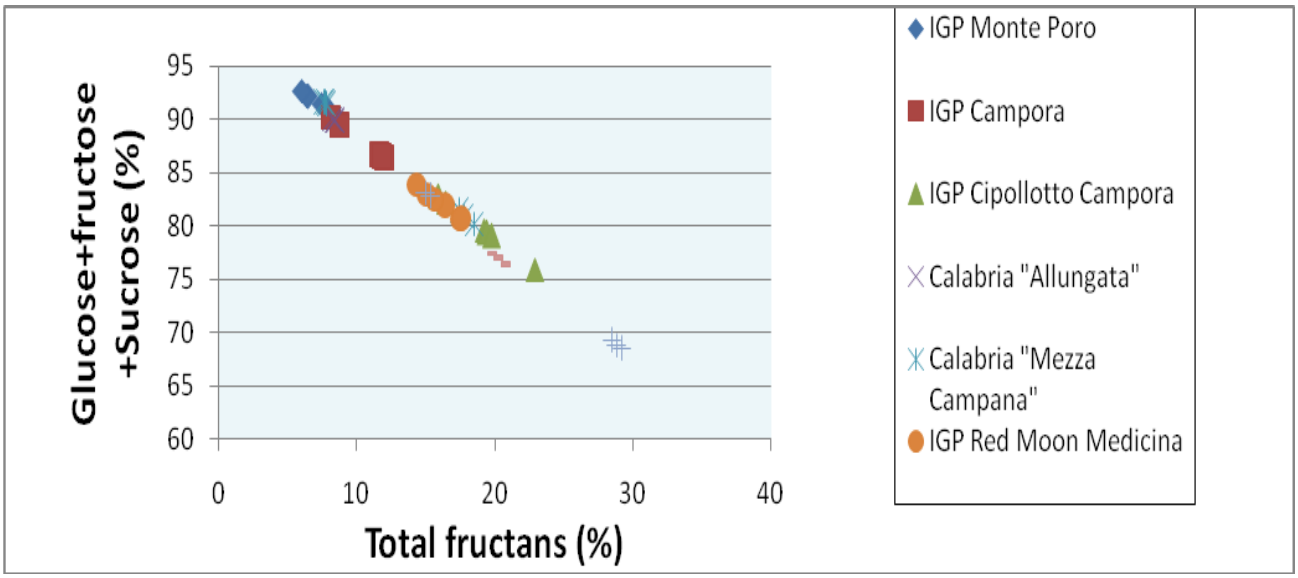

Figure 17.Correlation between total Fructans and sugars (r= 0.997;y=-1.04x+99.04).

8.3.9 Chemometric analyses

Initially a PCA analysis was carried out on 78x 45 , where 78 was the number of onion samples (three for each caliber of the same cultivar), of three colour skins: red, yellow and white onions and 45 the number of variables that correspond to all peaks detected in the chromatographic profile, also the not-identified ones corresponding to FOS with DP>5 and IOS. In Figure 18 it is reported the scree plot about eigenvalue. Computation of the PCs resulted in the first and second principal components described 23.22 and 13.52% of the variability in the original observations respectively. Table 11 shows the component score coefficient matrix .

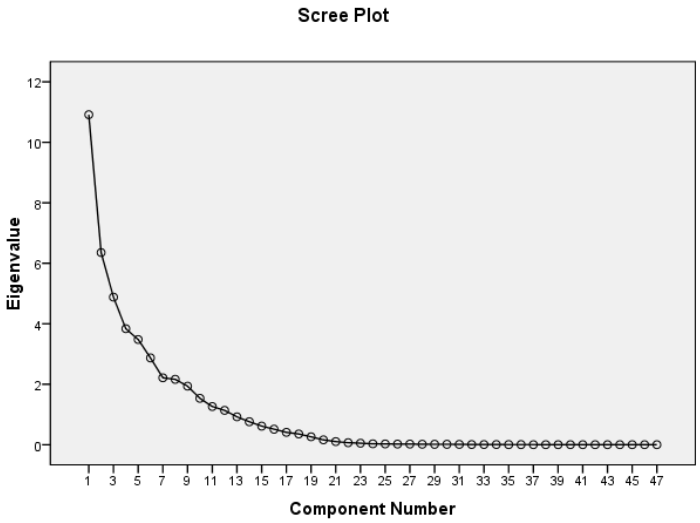

Figure 18. Scree plot

Figure 19 shows the sample score in the space defined by the two first principal components. As it can be seen, onions are principally discriminated by cultivar, with some differences between caliber, as previous observed.

Table 11. Component score coefficient matrix

	Component1	Component2
Glucose	,011	,121
Fructose	,009	,126
Peak 3	,055	,080
Sucrose	,027	,090
Peak 5	,042	,004
Peak 7	,001	,076
Peak 8	-,044	,041
Kestose	,012	,001
Peak 9	-,032	,083
Peak 10	,055	,069
Peak 11	,013	,000
Peak 12	,038	-,008
Peak 13	-,003	-,070
Peak 14	,014	,038
Peak 15	-,022	,016
Peak 16	,009	,090
Nystose	,032	-,081
Peak 18	,006	-,052
Peak 19	,067	-,024
Peak 20	,018	-,116
Peak 21	,028	-,068
Peak 22	,045	,082
Peak 23	,034	-,111
Fructofuranosyl-nystose	,071	,001
Peak 24	,043	,042
Peak 25	,014	,021
Peak 26	,045	-,041

Peak 27	,023	,010
Peak 28	,068	,007
Peak 29	,006	-,034
Peak 30	,040	-,074
Peak 31	,069	,049
Peak 32	,071	-,007
Peak 33	,040	-,081
Peak 34	,051	-,013
Peak 35	,038	-,039
Peak 36	,031	-,026
Peak 37	,060	-,070
Peak 38	,059	,041
Peak 39	,072	,016
Peak 40	,075	,020
Peak 41	,053	,010
Peak 42	,072	,004
Peak 43	,038	-,006
Peak 44	,059	,003
Peak 45	,054	,009
Peak 46	,043	,047

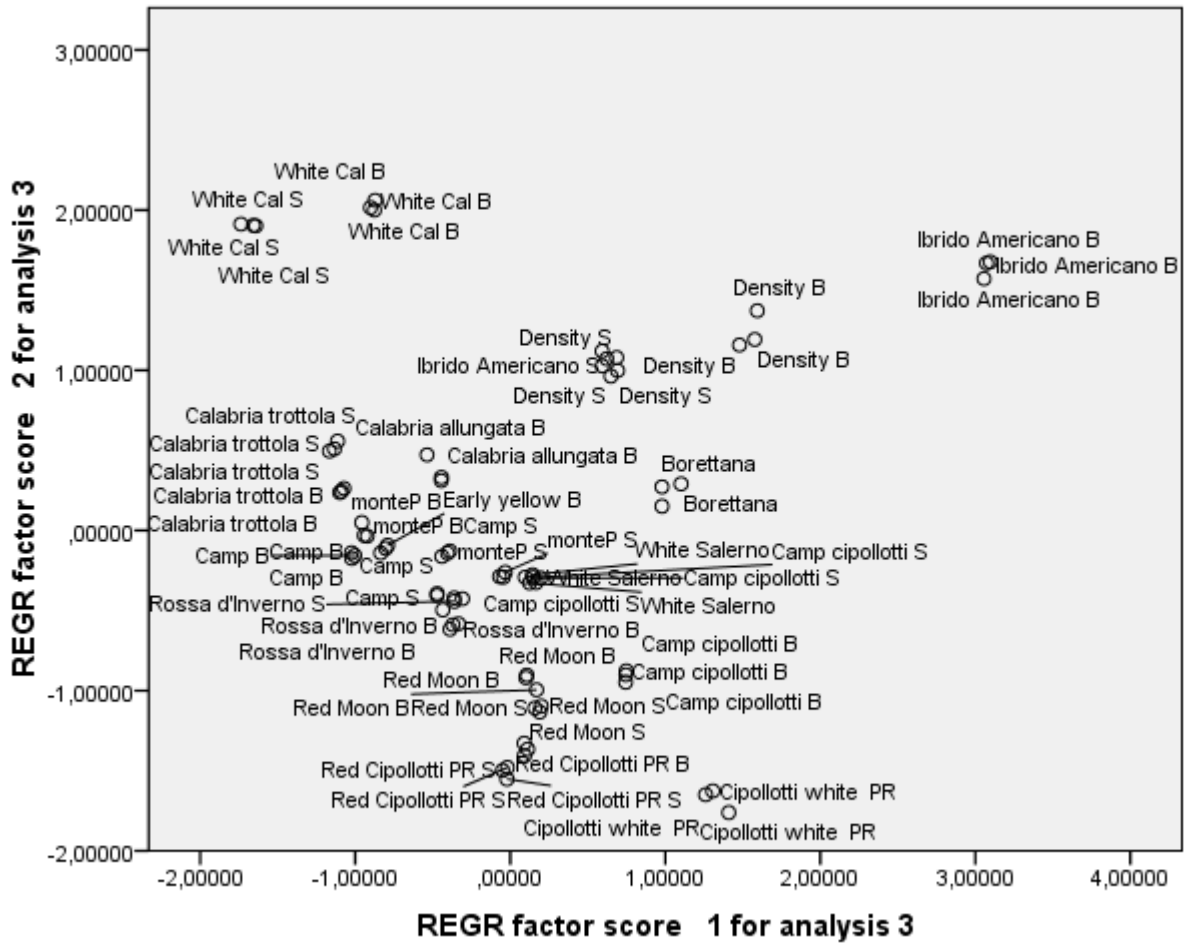

Figure 19. PCA score plot of onions profile of different colour skins from different geographical orgin

With the aim to verify if PGI Allium cepa var. tropeana chromatographic profile discriminated this onion from the others red ones, a Discriminant Analysis (DA) was performed.

Pearson correlation coefficients are calculated about all peaks detected and firstly focused our attention on glucose, fructose, sucrose and FOS DP 3-5 that are normally identified and quantified in fructans HPAEC-PAD analyses.

Pearson product moment correlations between each pair of variables range between -1 and +1 and measure the strength of the linear relationship between variables. The number of pairs of data values used to compute each coefficient was 78. The P-values, which tests the statistical significance of the

estimated correlation, lower than 0.5 indicates statistically significant non-zero correlation and are: glucose with respectively fructose, sucrose, nystose and fructofuranosylnystose; fructose with glucose, sucrose, nystose and fructofuranosyl-nystose; sucrose with nystose, and fructofuranosyl-nystose.

In a previous work, Kahane et al, 2001, discriminated onions from the following parameters: dry matter, fructose, glucose, sucrose, fructans with DP3-6 and total content of fructans calculated by enzymatic AOAC analyses. They found that glucose was the most important trait to distinguish accessions from each other, fructose was the most sensitive to environmental factor, sucrose and 1-kestose are the transient molecules (from transport to fructan build-up) rather than storage compounds. The total content of fructans resulted to have an high coefficient for discriminating onions but no notices about the single role of oligosaccharides with DP higher than 6 are reported. In our work the optimization of HPAEC-PAD method had permitted to obtain a good separation of these oligosaccharides and all peak areas are integrated to the aim to perform chemometric analyses.

In Table 12 discriminant functions, that a linear combinations of the input variables used to separate data into different groups, are reported as standardized and unstandardized coefficients. It is possible to observe that between FOS and sugars normally quantified and identified only fructofuranosyl-nystose showed an high value of standardized coefficient (major than 3), in the Function 2. This consideration remarks the importance to consider also peaks with DP higher than 5 for discriminate analyses.In particular, Peak 25 resulted to have the highest coefficient for the function 2 (value: 14.10) while peak 28 have the highest coefficient for the function 1(value 14.15).

These peaks are identified in the chromatogram reported in Figure 20 and the relative discriminant functions plot was showed in Figure 21. It is possible to observe the discrimination between Tropea onions labeled with PGI indication and other red onions , both from Calabria and Emilia-Romagna. Obviously this model has to be tested during a longer times, performing on the same cultivar cultivated in different years, but it could be an interesting approach, for example, to valorize onions with protected geographical origin and, by a discrimination from the others onions, to prevent the most frequent fenomenous of fraud.

Table 12. Discriminant functions standardized and unstandardized coefficients

Standardized Coefficients

	1	2
glucos	-0.596383	-0.206474
p3	-0.285959	-2.11852
p5	2.29403	-7.5104
p7	3.61579	1.13268
p8	0.291851	9.17609
kestos	0.56802	-0.162848
p10	2.13878	-8.50093
p11	-3.91024	-7.56565
p12	0.337867	-0.749577
p13	0.773404	4.44593
p14	6.06925	-11.2383
p15	-9.74672	9.42601
p16	0.512926	2.69193
nistos	1.88447	-2.25987
p18	7.0272	-6.56861
p19	1.54426	-1.87806
p20	6.06723	-1.77379
p21	8.71535	-3.40192
p22	-3.65979	5.22536
p23	6.39017	8.78196
fruttofur	2.64197	3.52547
p25	-3.96675	14.1029
p26	1.23838	9.38188
p27	-12.388	7.06081
p28	14.1497	3.3205
p29	-4.06478	-1.21727
p30	3.73875	2.05535
p31	-0.34893	8.62483
p32	0.88683	1.81104

Unstandardized Coefficients

	1	2
glucosio	-0.0125407	-0.00434173
p3	-0.0818112	-0.606097
p5	4.01618	-13.1485

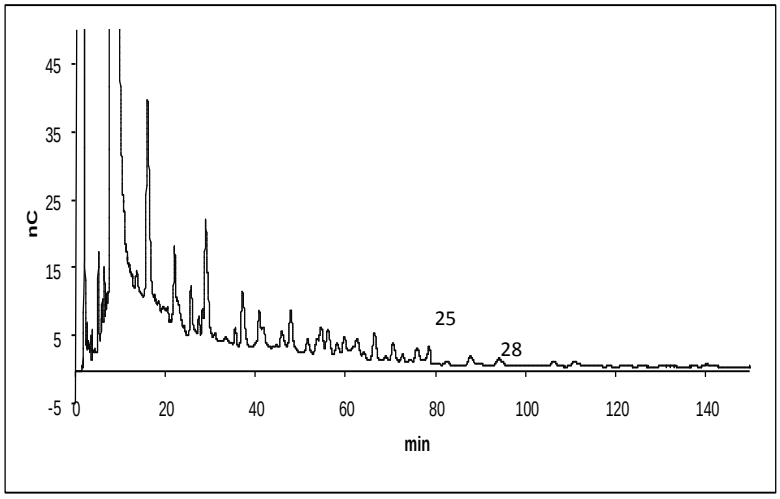

Figure 20. Peaks 25 and 28 identified in the chromatogram

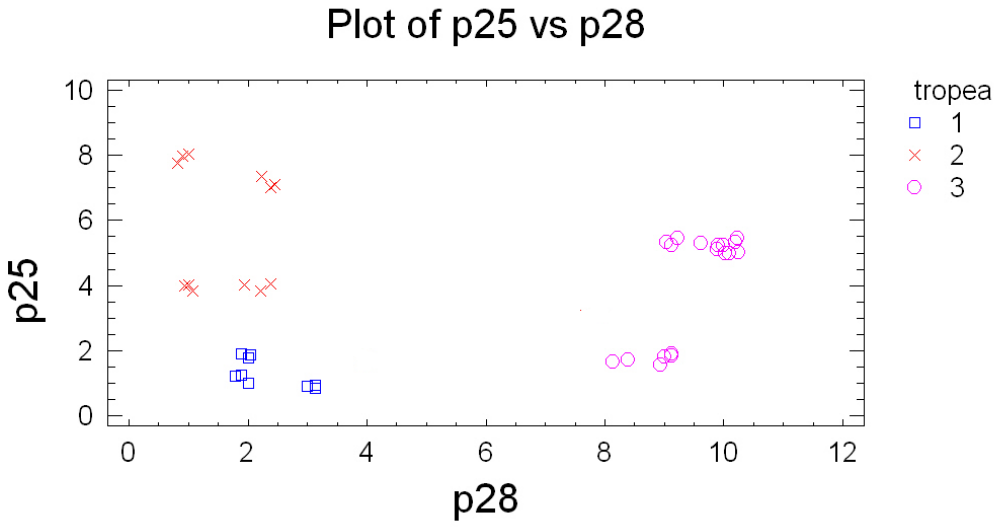

Figure 21. 1= Calabria red onions without PGI indication (onions are sub grouped in two shapes and in small and big caliber) .2= IGP var. tropeana from two geographical area: one nearer to sea, "Campora S.Giovanni" (Cosenza) and one "Monte Poro"(Vibo Valentia, on the mountains) and sub classified in small and big caliber, 3-Other red onions (Emilia-Romagna onion

3.10 Acknowledgments

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9. DETERMINATION OF NITRATE AND NITRITES IN VEGETABLES AND IN COMMERCIAL CARBOHYDRATES

9.1 Introduction

9.1.1 Fructans safe intakes

Inulin and oligofructose are a significant part of the daily diet of most of the world's population. Daily intakes for the U.S is estimated between 1 and 4 g and in Europe between 3 and 11 g. Therefore it is important to evaluate their safety, though it is difficult to apply classical toxicology tests. Numerous studies (Roberfroid, 1993) document that providing additional assurances of inulin and oligofructose both to normal subject and patients with diseases states, there is not toxic effect on the health and, on the contrary a prebiotic effect is recognized (Kolbye et al, 1992). Several studies in vitro shown the absence of a mutagenic or genotoxic potential. Furthermore inulin and oligofructose are legally classified as food or food ingredients and not as additives in all countries in which they are used (Coussement, 1999) . In the U.S., a committee of experts declared both inulin and oligofructose as Generally Recognized As Safe (Kolbye et al, 1992). In European countries no ADI were fixed and these substances are generally accepted for food use without limitations. The recognized negative effects related to high doses of fructans, are the increase of flatulence and osmotic pressure. A series of clinical studies has been reported that up to 20g/day of inulin is well tolerated (Carabin and Flamm, 1999). The Australian food government in 2008 (Application A609) permit the voluntary addition of inulin-derived substances and galactooligosaccharides (GOS) to infant formula products, infant foods and formulated supplementary foods for young children (FSFYC) with a maximum of 110 mg per 100 kJ (3 g/L); while it does not permit the addition of fructo-oligosaccharides (FOS), to these foods for insufficient evidence to support.

9.1.2 Industrial processes to obtain sugars and fructans

The sugars extraction processing (explained in Figure 1) starts by slicing the beets into thin chips. This process increases the surface area of the beet to make it easier to extract the sugar. The extraction takes place in a diffuser where the beet is kept in contact with hot water for about an hour. The diffuser is a large horizontal or vertical agitated tank in which the beets slices slowly work their way from one end to the other and the water is moved in the opposite direction. An higher sugar concentration solution was obtained, named juice. The exhausted beet slices from the diffuser are still very wet and the water in them still holds some useful sugar. They are therefore pressed in screw presses to squeeze as much juice as possible out of them. This juice is used as part of the water in the diffuser and the pressed beet, by now a pulp, is sent to drying plant where it is turned into pellets which form an important constituent of some animal feeds. The juice is as carbonated: a small clumps of chalk are grown in the juice. The clumps, as they form, collect a lot of the non-sugars so that by filtering out the chalk one also takes out the non-sugars.

Once this is done the sugar liquor is ready for sugar production except that it is very dilute . Therefore the juice is evaporated in a multi-stage evaporators. In the last stage, the syrup is placed into a very large pan, typically holding 60 tons or more of sugar syrup. Once the crystals have grown the resulting mixture of crystals and mother liquor is spun in centrifuges to separate the two, rather like washing is spin dried. The crystals are then given a final dry with hot air before being packed and/or stored ready for dispatch.

Inulin and FOS extraction process are resumed in Figure 2 and best explained in the Chapter 5.

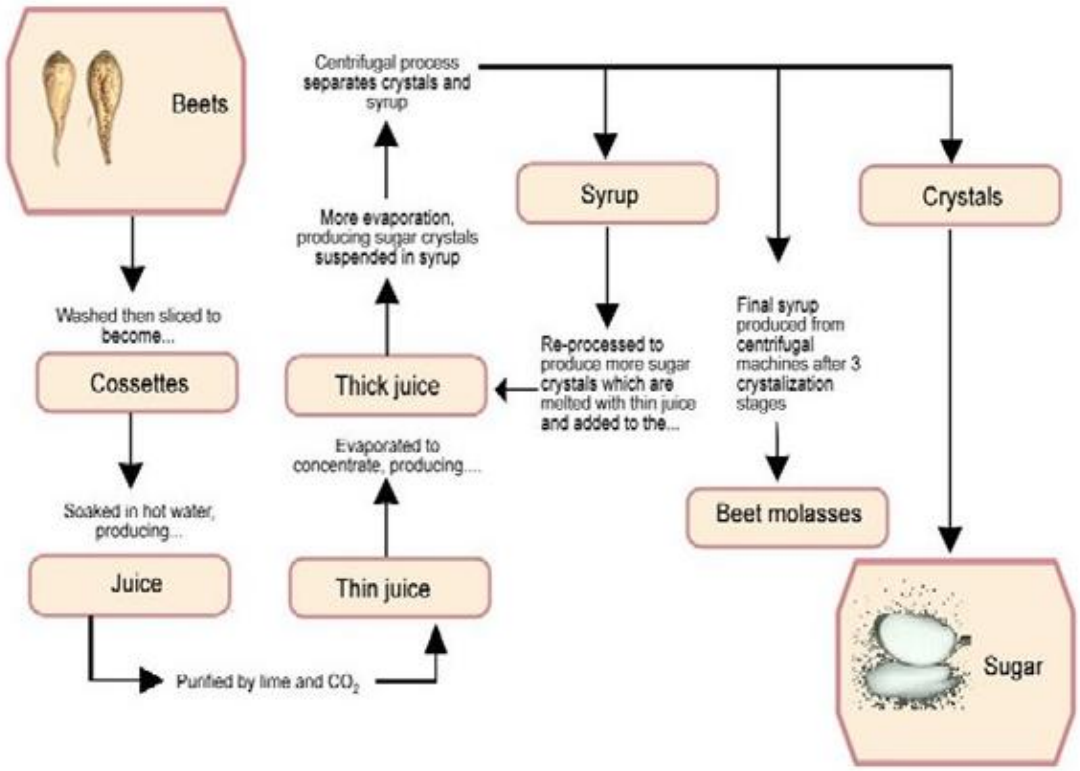

Figure 1- Sugar production industrial process

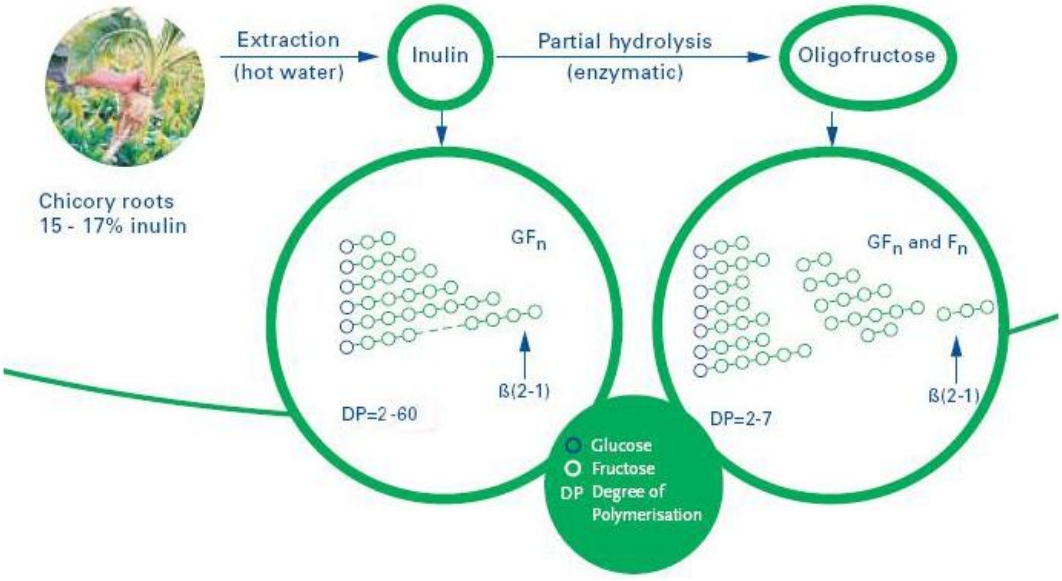

Figure 2- Inulin and FOS extraction process

9.1.3 Nitrates and nitrites

Nitrate is a common chemical compound in the nature, and is widely found in soils, waters, and foods. The daily nitrate intake occurs from three major sources: vegetables, drinking water, and meat products.

Although great differences exist in diary habits and water quality between different countries, vegetables are still the major source of nitrate intake. They constitute 30-90% of the total nitrate intake. An Acceptable Daily Intake (ADI) for nitrate of 3.7 mg/kg b.w./day, equivalent to 222 mg nitrate per day for a 60 kg adult was established by the former Scientific Committee on Food (SCF) and was reconfirmed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2002. The CONTAM Panel noted that no new data were identified that would require a revision of the ADI. As a conservative base case, a person eating 400g of mixed vegetables at typical median nitrate concentration levels would on average receive a dietary exposure to nitrate of 157 mg/day. This is within the ADI even when the exposure to nitrate from other dietary sources is considered. Considering that for most people, fruit, which has low nitrate levels in the order of 10 mg/kg, comprises up to one half of the total recommended daily intake of 400 g of vegetables and fruit, actual nitrate intakes would be reduced to between 81-106 mg/day for the majority of the EU population. Further mitigation of nitrate intake may result from processing e.g. washing, peeling and/or cooking. (EFSA journal 2008, 689).

Classification of vegetables according to nitrate content (mg/kg FW) was proposed by Santamaria, 2006 and resumed in Table 1.

Table 1.Classification of vegetables in accord to their nitrate content

Nitrate content (mg/kg FW)	Vegetable varietes
Very low (<200)	Artichoke, asparagus, broad bean, eggplant, garlic, onion, green beat, mushroom, pea, pepper, potato, summer squash, sweet potato, tomato, watermelon
Low (200-500)	Broccoli, carrot, cauliflower, cucumber, pumpkin, "puntarelle"chicory
Middle (500-1000)	Cabbage, broccoli, dill, savoy, cabbage, turnip
High (1000-2500)	Celeriac, chinese cabbage, endive, fennel, kohrabi, leek, parsley
Very high (>2500)	Celery, cress, chervil, lettuce, red beetroot, spinach, rocket

As the vegetables first enter into the body by swallowing, the conversion of nitrate in the mouth is particularly important. The dorsal surface of the tongue symbiotically harbors a specialized flora of anaerobic nitrate reducing bacteria, which can rapidly reduce nitrate to nitrite, hence, the contents of nitrite in saliva are generally higher.

Because of the absence of nitrite reductase in the oral cavity, nitrite will convert to varieties of nitrogen compounds in the stomach. The main products are NO and N-nitroso compounds (NOCs) include nitrosamine and nitroamide.

Generally, it is considered that high nitrate intake increased the risk of cancer and methemoglobinaemia, even hyperparathyroid, children polyuria, hypertension, and so on (McKnight et al. 1999). However, in recent years, many researchers found that the nitrate has lots of beneficial effects on human health.

Nitrate is recycled in the blood, concentrated by the salivary gland, and repeatedly recirculated through the stomach rather than being secreted immediately, suggesting the beneficial physiological function which is attributed to the metabolite of nitrate.

9.1.4 Aim of this work

The first aim of this work was to test the security of some carbohydrates and fructans, object of this thesis, by analysis of nitrates. They have never been considered as possible source of nitrate intake although Mack, Hoffmann, & Marlander (2007) reported a study on sugar beet with data about the quantification of different forms of soluble nitrogen compounds, including nitrate.

Analysis of nitrates are extended to some onion samples, previously studied in the Chapter 8, as natural source of fructans, and to other vegetables samples like spinach, carrots, chard.

On spinach samples a study of the effects of blanching and freezing on spinach was conducted, verifying if the technological treatments (blanching, freezing, cooking) on vegetables can affect nitrate content.

These analyses are performed by capillary zonal electrophoresis (CZE) , a powerful technique for anions analyses, with the advantages to obtain low running cost.

In particular, a new CZE method for the simultaneous analysis of nitrate, nitrite and oxalate was optimized.

9.2 Materials and methods

9.2.1 Commercial Standards and samples

The nitrate, nitrite and oxalate stock solutions were prepared separately for each analyte at 5 gL^{-1} levels in deionized water and stored at 4°C. The stock solutions were gradually diluted at the working concentration levels with background electrolyte. All stock solutions were filtered through a 0.22 μm membrane filter (Siringe filters Teknokroma).

Different source of fibers were analyzed: Beneo® P95, Beneo® GR, Beneo® HP (Orafti, Tienen, Belgium); Frutafit® IQ and Frutafit® TEX (Sensus, Roosedaal, The Netherlands); Fibruline Instant, Fibruline XL (Cosucra, Belgium). Vegetables, sugar table and dextrose have been purchased from local market; except that Medicina and Tropea onions (see Chapter 9), deionized grape juice concentrate that was obtained from Caviro distillerie, Italy and sucrose (Sigma Aldrich).

Vegetable samples analyzed are:

Spinach	fresh (leaves)	
	fresh (stalks)	
	ready-to-eat	
	frozen	
Chard	fresh (leaves)	
	fresh (stalks)	
	frozen	
Carrots	fresh	
	Ready-to-eat	
Red onions skin (fresh)		PGI MontePoro (n=3 for each caliber, as described in the Chapter 8)
		PGI Campora (n=3 for each caliber)
		PGI Campora cipollotti (n=3 for each caliber)
		PGI Red Moon (n=3 for each caliber)
		PGI Rossa d'Inverno (n=3 for each caliber)
		Calabria allungata (n=3 for each caliber)
		Calabria trottola (n=3 for each caliber)

9.2.2 Sample extraction

A- Table sugars and dietary fibers

The solid Phase Extraction technique (SPE) was employed for nitrates extraction from table sugar and dietary fibers samples, using quaternary amines solid phase on silica support. The cartridge are firstly conditioned with 2 mL of methanol and 2 mL of deionized water, then 6 mL of sample (previously diluted 1:33 with deionized water), was loaded and eluted with 2 mL of sodium hydroxide 1 M, flow rate 0.5 ml/min. The final sample solution was diluted 1:1 (v/v) with background electrolyte (BGE) and adjusted at pH 7.0 with phosphoric acid 9 M. All samples and standards were filtered through Nylon syringe-type filter, 0.2 μm porosity before analysis.

B- Vegetables

Regarding vegetables, nitrates and nitrites were extracted from matrices by homogenization with deionized water in a ratio 1:40 for all vegetables except than onions where the ratio was 1:50. Then solutions were heated at 100 °C during 15 minutes, under continuous agitation, followed by buckner filtration (Farrington et al, 2006) and then by the Nylon syringe-type filter of 0.2 μm porosity. Total oxalates were extracted with hydrochloric acid 2N (in a ratio 1:2) according to the procedure reported by Honow and Hesse, 2002, to extract even the amount of oxalates complexed to inorganic cations naturally occurring in the matrix. All samples and standards were filtered through nylon syringe-type filter, 0.2 μm porosity before analysis.

For all samples, the dry matter was performed keeping the samples in the oven at 102°C; weight measures were repeated every six hours until the samples reached a steady weight loss, except for onions samples, which method was which reported by Kahane, et al, 2001 .

9.2.3 CZE Apparatus

The analyses were conducted by A Model P/ACE MDQ electropherograph with UV detector (Beckman Coulter™, Fullerton, CA, USA and 32 Karat™ 5.0 software). Separations were carried out under these conditions:

-Untreated fused-silica capillary (Beckman Coulter™ and Restek, 110 Benner Circle, Bellefonte, Pennsylvania, USA) of 75 μm i.d., effective length of 21.5 cm, total length 32.5 cm.

-Electrolyte: Phosphate buffer (50 mM), pH 2.5.

-Reversed polarity

-Capillary

-Voltage:-25 kV

-Temperature:25°C

-Capillary pressure injection: 3.5 kPa for 5 sec

-UV detection .214 nm

All electrophoresis runs were done in triplicate.

Before use, new capillaries were conditioned with methanol for 2 min, water for 1 min, 0.1 N chloridric acid for 2 min, water for 1 min 1 M sodium hydroxide for 5 min, water, and the background electrolyte for 20 min using a pressure major than 150 kPa.

9.3 Results and discussion

9.3.1 Optimization of CZE method

Anions injected onto the capillary move towards the anode because of their negative charge.

Since electroosmotic flow (EOF) is normally directed to the cathode, and therefore in the opposite direction, it is convenient to reverse it allowing anions co-migration.

EOF strength can be modulated by action on several parameters with the aim of neutralizing the negative charges of silanol groups on capillary wall.

On other hand, reversal of the EOF direction in fused-silica capillaries occurs when specific adsorption of counter ions in the immobilized region of the double layer takes place (Jandik and Bonn, 1993).

This goal is often reached by addition of surfactants to the buffer by chemical coating of the capillary (Mo, et al, 2008; Talaván García, et al, 2007, Noblitt, et al, 2007).

In this work it was conducted a parallel studies on the mobility of uncoated EOF capillaries, in comparison with the coated capillary. Different types of uncoated capillary where examined on which tests were performed with increasing electrolyte pH values from 2.0 to 9.0, maintaining a constant ionic strength during all tests.

For each ball of capillary considered two samples were taken of total length 30.1 cm and effective length 21.1 cm; each capillary was activated under the same conditions by performing alternating washes with hydrochloric acid, water and sodium hydroxide 1M.

As EOF marker it was employed mesityl oxide, that is molecule non ionizable at values of pH considered; each analysis was conducted in triplicate. In Figure 3, it is reported the dependence of EOF on pH of two of uncoated capillary examined.

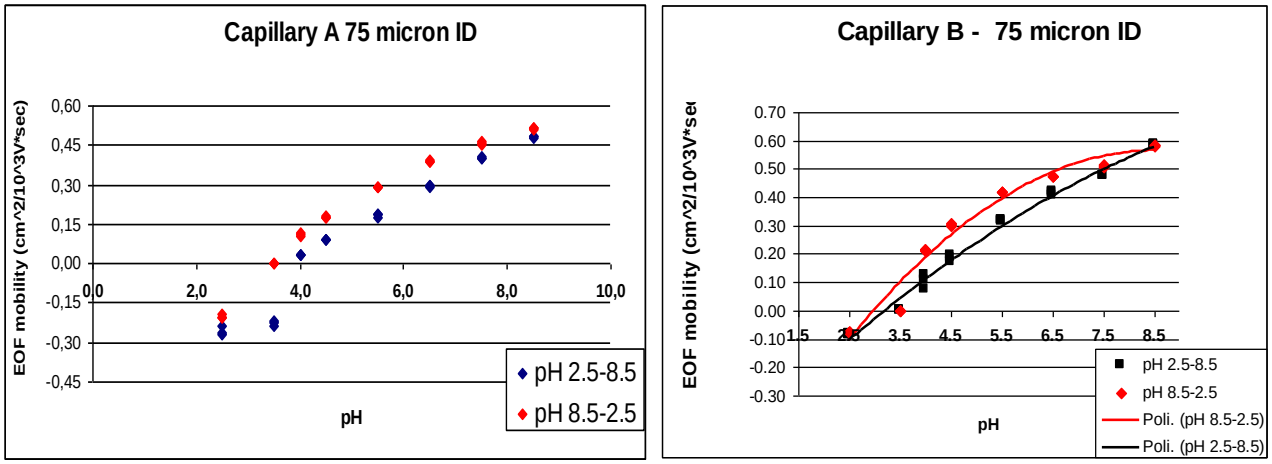

Figure 3. Curves representing the trend of EOF mobility as a function of pH of electrolyte solution at constant ionic strength.

As it is possible to observe from Figure 3, at pH below 3.5, after an intensive rinse, silanol groups of the inner surface of the silica-based capillary assume a positive charge and EOF is reversed from cathodic to anodic. Under these conditions, no additive or any treatment of capillary is requested and anion mobility is increased by an anodal EOF.

The pH and the ionic strength of the background electrolyte (BGE) were optimized for nitrates and nitrites separation. The use of a BGE consisting of 50 mM phosphate buffer at pH 2.5 permitted a good resolution of all analytes. Under these conditions, nitrate eluted before nitrite; because at lower and Lucy, 2000). The order of migration, as explained by electropherogram in Figure 4, was nitrate, oxalate, nitrite and EOF marker, whose presence confirms that EOF was reversed.

The electrophoretic method was validated following EURACHEM guides, 1998, on a standard solution. LOD values were 0.3 mg/L for nitrate, 0.8 mg/L for nitrite and 25 mg/L for oxalate, while LOQ values were respectively 0.5 mg/L, 1.6mg/L and 30 mg/L. Linearity was established over two orders of magnitude of concentration, differing for each standards (nitrate: 0.5-100 mg/L; oxalate: 30-700 mg/L; nitrite: 1.6-160mg/L), at six equi-spaced concentration levels performing three replicated injections at each level. All R^2 values were higher than 0.993.

Intra-day and inter-day repeatability were calculated, on apparent and effective mobilities, injecting respectively the samples ten times in the same day and on three non-consecutive different days.

Precision of concentrations was calculated on two concentration levels (5 and 20 mg L-1): the RSD% was $\leq$ 2% for intra-day analyses and $\leq$ 4% for inter-day analyses for all analytes in standard solution and samples.

Recovery percentages were calculated both on two samples of spinach fortifying with the analyte, each at two different concentrations, corresponding to 10 and 70% and adding 5 ppm of nitrate to a onion sample. Average of recovery percentages were higher than 90%.

Quantitative analyses were carried out by using an external standard calibration method.

Calibration curves were determined for six different concentrations of analytes standard solution, injecting each level of concentration in triplicate. All correlation coefficients of the calibration plots are greater than 0.994.

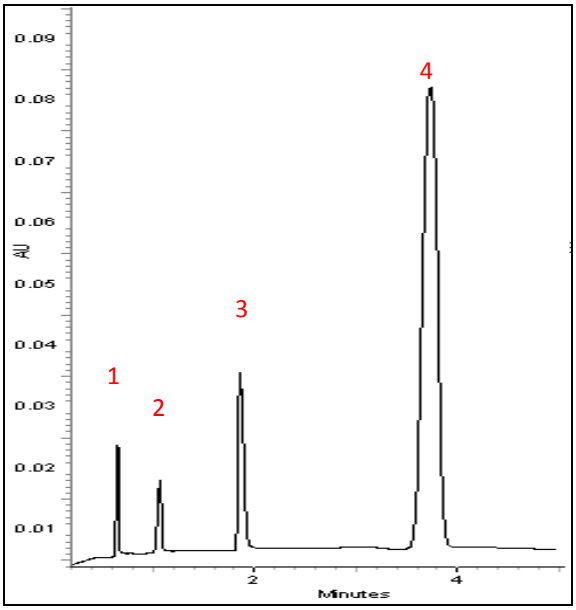

Fig.4 Electropherograms of mixture of standards :1.Nitrate; 2. Oxalate; 3. Nitrite; 4. EOF marker.

9.3.2.Determination of nitrates in some commercial carbohydrate samples

All sugars (white, cane beet and grape sugars) and fructans (both inulin and fructooligosaccharides) showed values of nitrate values higher than limit of quantitation, while nitrites and oxalates are not detected (Figure 5). Regarding sugars analyses, refined and cane sugars have a similar nitrate content (in a range between 4.87 and 6.85 mg/kg) while sugar derived from grapes contains a lower amount of nitrates (0.94 mg/kg) since it doesn't derive from vegetables roots. Regarding fructans analyses, many differences are observed relate to the degree of polymerization. This is could explained considering the industrial extraction process of fructans extraction: fructooligosaccharides are obtained from inulins after a physical or enzymatic process, followed by a purification step.

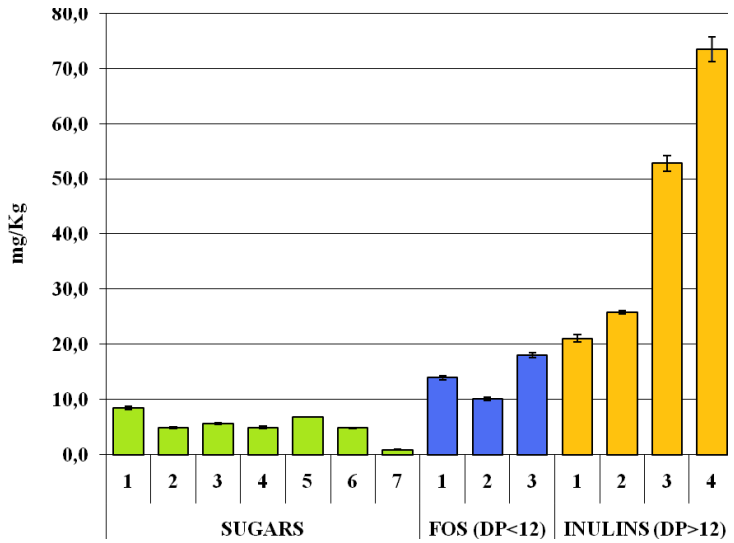

Figure 5. Nitrate content in table sugars and in fructans samples. Sugars: 1-Sucrose, 2-Dextrose, 3-Sugar beet A, 4-Sugar beet B, 5-Refined sugar cane, 6- Raw sugar cane, 7-Grape sugar; FOS.1-Beneo P95, 2-Fibruline Instant, 3-Beneo GR; Inulins: 1-Frutafit TEX, 2-Frutafit IQ, 3-Beneo HP, 4-Fibruline XL.

9.3.3. Determination of nitrates, nitrites and oxalate in some commercial vegetable samples

Both leafy vegetables (spinach , *Spinacia olearia L* and chard, *Beta vulgaris L*), carrots (*Daucus carota L.*) and onions (*Allium cepa L.)* are analyzed to verify the presence and the content of nitrates, nitrites and oxalate. Nitrites values are under LOQ for all vegetables analyzed, while oxalates are not detected in carrots, in onions , in accord with literature (Jaworska b, 2005; Conte et al., 2008).

In the spinach samples nitrates (expressed as g/kg dry matter) the oxalates are in a minor concentration in frozen samples and in a major quantity in ready-to-eat-spinach (Table 2).

In spinach and chard stalks oxalates values are under LOQ, confirming that oxalates accumulate chiefly in leaves (Kabaskalis, 1995).

Regarding nitrates, values (g/kg dry matter) are very different regarding the family of the vegetable but also in relationship with the technological treatment (Table 3).

Table 2. Oxalate (g/kg D.M.)in vegetables

Vegetable samples		g/kg dry matter ±SD
Spinach	Fresh (leaves)	210.65±8.38
	Ready-to-eat	235.40±3.41
	Frozen (sample A)	100.95±4.13
	Frozen (sample B)	107.51±2.34
	Frozen (sample C)	159.28±1.61
Chard	Fresh (leaves)	149.75±2.75
	Frozen	148.91±1.38

In accord to classification of Santamaria, 2006, regarding fresh samples, onions have the lower content of nitrate, with a mean value of 50.80 mg NO_3^- /kg FM, followed by carrots (243.01 mg NO_3^-/kg FM) , spinaches (leaves: 1272.12 mg NO_3^-/kg FM; stalks: 8557.23) and chards (leaves: 2380.14 mg/ NO_3^-; stalks: 9090.34 mg/ NO_3^-).Contrary to oxalates, in accord to Jaworska, 2005a, spinach and chard stalks contained more nitrates than leaves.

Variability between spinach frozen samples (between 23.98 and 30.67 g/kg DM) can be attributed to the differences in the leaves: stalks ratio, which depends on the quality of the frozen product. For this reason it is not easy to compare fresh samples to frozen samples, but it is previously reported that the blanching process reduce nitrate content (Jaworska, 2005b).

Ready-to-eat products (spinach and carrots) contain fewer nitrates than fresh ones, due to technological treatment.

Table 3. Nitrates (g/Kg D.M.)in vegetables

Vegetables sample	Type	g Kg^{-1} dry matter ±SD
Spinach	fresh leaves	17.59±0.58

	fresh stalks	118.29±2.90
	ready-to-eat	10.41±0.66
	Frozen (sample A)	30.67±0.06
	Frozen (sample B)	23.98±0.07
	Frozen (sample C)	24.31±0.11
Chard	fresh leaves	15.47±0.03
	fresh stalks	88.76±0.15
	frozen	33.49±0.52
Carrots	fresh	1.98±0.07
	ready-to-eat	0.81±0.02
Red Onions (fresh samples)	PGI Monteporo B	0.67±0.05
	PGI Monteporo S	0.75±0.03
	PGI Campora B	0.98±0.03
	PGI Campora S	1.24±0.02
	PGI Campora cipollotti S	0.07±0.01
	PGI Campora cipollotti B	0.09±0.02
	Calabria allungata B	0.01±0.00
	Calabria trottola B	0.01±0.00
	PGI Red Moon B	0.06±0.00
	PGI Reed Moon S	0.07±0.00
	PGI Rossa d'inverno B	0.06±0.00

Regarding onion samples (Figure 6), differences in nitrate content are noticed between different cultivars, geographical origins, calibers. Nitrates concentration in onions is strictly related to the type of the soil and the fertilization techniques. For example *Allium cepa tropeana* samples cultivated in Campora San Giovanni area have a major nitrate content than the same samples cultivated in Monte Poro area. As previously reported in the Chapter 9, Campora San Giovanni soil, near to the sea, is principally composed of sandstone and clay; then fertilization is more abundant and frequent respect than the medium-textured Monte Poro soils, collocated at an altitude of 710 m. This observation was noticed also for the dry matter content (Chapter 8), confirming an inverse relationship between dry matter percentage and nitrate content, as reported by Santamaria et al, 1999. Cipollotti have lower nitrate values than the respective onions, probably because they are harvested before maturation and then they stay in the ground for a minor number of months. Considering the two caliber: small (generally diameter: 40-60 mm) and big (generally diameter: 60-80 mm), all onions with a small caliber have lower values of nitrates. Probably it is due to the fact that plants produce the enzyme nitrate reductase, which converts organic nitrogen into nitrate nitrogen, and then in enzymes and proteins used for plant growth. It possible that the greater concentration of nitrate is related to a minor growth of the onion as a consequence of a lower use of nitrate nitrogen.

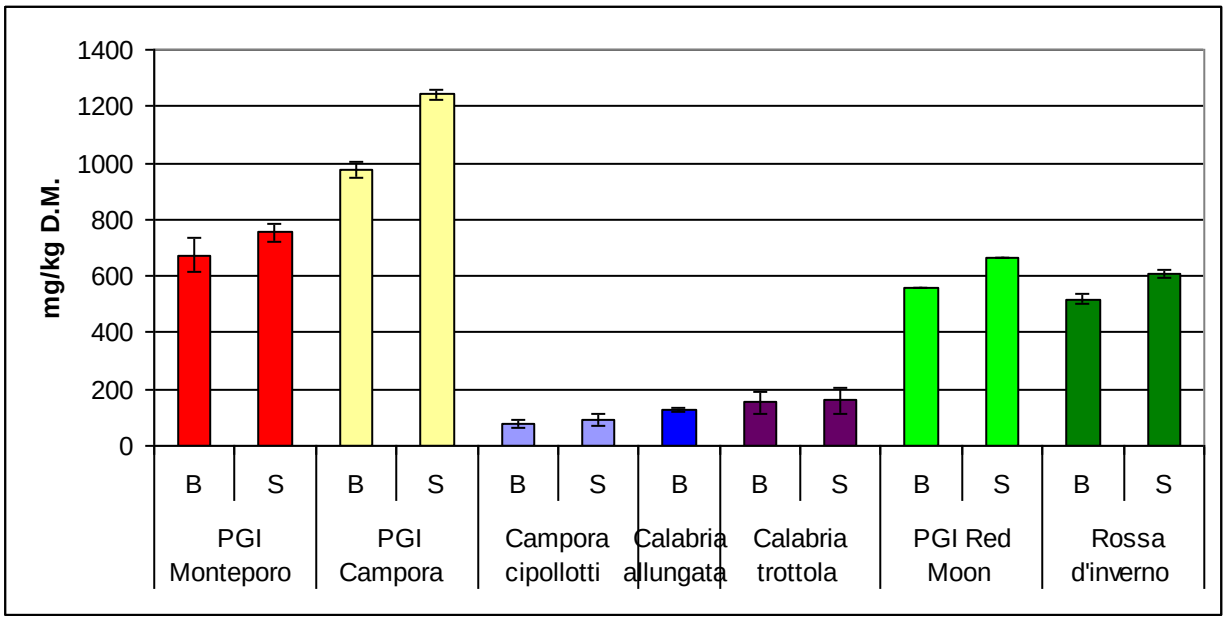

Figure 6. Nitrate values (mg/Kg D.M.) of red onion samples

9.4 Conclusions

The appreciable amounts of nitrates in carbohydrates and in soluble fiber is an important aspect to evaluate in the formulation of functional foods and babyfoods. Prebiotics ingredients as inulin and fructooligosaccharides are added to foods in which nitrates are already contained as for example hams or yogurt with fruits. This study has been possible by the optimization and the validation of an electrophoretic method that had permitted to obtain fast and reliable analyses, and it could permit the analyses of nitrates, nitrites and oxalates in many foods. The major advantage of this method is the possibility to reverse EOF without dynamic coating, but only with the employment of an acid buffer.
Our analyses conducted on different types of vegetables has confirmed that nitrates content it is not only dependent on the vegetable family but also it is affected by fertilization and irrigation techniques.

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This PhD thesis concerns the development and validation of high performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) methods for the analysis of carbohydrates of food interest. The choice of the columns, phase mobile and the optimization of gradient of elution for each carbohydrate studied had permitted to obtain good peaks resolution and separation both of simple sugars and complex carbohydrates in many food matrix.

For example by the optimization of an HPAEC-PAD method, it has been possible to study the metabolism of *Saccharomyces cerevisiae* inoculated on a substrate of *leguminosae* panel and to consider this a good alternative substrate to molasses.

In pasta samples, maltulose was separated from others carbohydrates, such as reducing carbohydrates that are involved in Maillard reaction, and it was found to be a good marker of pasta drying process quality in association with others parameters like furosine and colour indices.

Subsequently, soluble fibers with prebiotic properties, named fructans, have been analyzed. For these carbohydrates the problem of the lack of commercial IOS and FOS standard with degree of polymerization higher than 5 was firstly overcomed by Mass Spectrometric analyses (in particular MALDI-TOF-MS) that had permitted to identify the DP of fructans. Then a study about the pulsed amperometric detector response, that was conducted in relationship with the degree of polymerization of the soluble dietary fibers analyzed, has allowed to perform quantitative analyses of fructans naturally present in foods (such as in onion) or added as functional ingredients (e.g. fermented milks and cooked ham). Chemometric analyses were also performed for the characterization of onions of different varieties and geographical origin by a chromatographic profiles fingerprinting discrimination, focusing particular attention to onions with Protected Geographical Indication.

Nitrate content of these onions was also determinated by CZE analyses, performing a rapid and reliable method that has the advantage to permitt anion analyses by reversing EOF without dynamic coating, with the only employment of an acid buffer.

Furthermore this method had permitted to investigate nitrates content in sugars and in soluble fibers, considering the fact that are they more often added to functional foods for their recognised prebiotic properties.

Accepted Manuscript

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2) **Chiara Borromei**, Maria Careri, Antonella Cavazza, Claudio Corradini, Lisa Elviri, Alessandro Mangia, and Cristiana Merusi, Evaluation of Fructooligosaccharides and Inulins as Potentially Health Benefiting Food Ingredients by HPAEC-PED and MALDI-TOF-MS. *International Journal of Analytical Chemistry*, 2009, Article ID 530639, 9 pages, Available on line.

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Submitted Manuscript

7) Cristiana Merusi, Antonella Cavazza, **Chiara Borromei**, Paola Salvadeo, Claudio Corradini Determination of phenolic compounds in red wine along fermentation process by a validated coelectroosmotic capillary electrophoretic method, *Journal of Agricultural and Food Chemistry*, Submitted.

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Proceedings and Congress Partecipation

Oral Comunication

a) Cavazza A., **Borromei C**., Corradini C., Bersellini C., Fochi A. Utilizzo di un prodotto come substrato derivato da matrici vegetali di Leguminose come substrato per la crescita di lieviti, *Atti del VII Congresso Nazionale di Chimica degli Alimenti*, Perugia 23-26 Giugno 2008, ISBN 978-88-86993-28-9.

b) **Borromei Chiara**, Corradini Claudio, Cavazza Antonella, Determination of fructooligosaccharides of prebiotic interest in onions (*Allium cepa*) from different cultivars, **Proceedings** of EuroFoodChem XV, Copenhagen, 5-8 Luglio 2009, ISBN 978-87-993033-3-5.

c) **C. Borromei,** A. Cavazza, C. Corradini, T. Vicchio. HPLC-PAD determination of fructooligosaccharides profile for characterization of onions from different botanical and geographical origin , XXIII *Congresso Nazionale Società Chimica Italiana*, Sorrento, 5-10 July 2009**.**

d) **Borromei Chiara**, Tutor Prof.Claudio Corradini, Development and validation of HPAEC-PAD methods for carbohydrates analysis in the control of food quality**.** *Proceedings of the 14Th Workshop on the Developments in the Italian PhD Research on Food Science Technology and Biotechnology*, Oristano, 16-18 settembre 2009, available on line.

e) **Chiara Borromei,** , Antonella Cavazza, Claudio Corradini, Marco Giannetto, Cristiana Merusi, Giovanni Mori, Determination of fructans and nitrates to characterize onions (Allium cepa) from different Italian origin by HPAEC-PAD and CZE techniques, *Proceedings on 4th International Symposium on Recent Advances in Food Analysis*, 4-6 November, 2009, Prague, ISBN 978-80-7080-726-2.

f) **C.Borromei,** M.Careri, A.Cavazza, C.Corradini, L.Elviri, A.Mangia, Comparazione dei metodi MALDI-TOF-MS e HPAEC-PAD per l'analisi di fruttani impiegati come prebiotici nel campo alimentare, 1° Workshop "Scienza al CIM:la parola ai giovani", 27 Novembre, Parma.

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Poster Comunication

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