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CICLO XXX

DEVELOPMENT OF ANALYTICAL METHODS FOR FOOD CHARACTERIZATION AND SECURITY EVALUATION

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PREFACE

Food security and *food safety* are two very important concepts, which can be correlated to food quality. F*ood security* is linked to the possibility to provide food and water to all people in hygienic conditions to satisfy the energy needs for humans' survival and life; *food safety* aims at obtaining safe food products since the latter are exposed to different hazards throughout the agri-food chain.

Food quality and food safety can be managed, for example, through the control of product and/or process molecular markers attesting quality and / or typical characteristics of food. Great importance have also all materials intended to come into contact with food (generally called Food Contact Materials) due to the possibility of accidental migration of substances into foodstuff.

Recently, the interest towards nanotechnology to obtain innovative packaging materials to extend products shelf life, has raised. An important focus regards nanomaterials in polymer matrices, since usually there are not sufficient data about possible interaction with foods and their effects on the human body.

Furtermore, social changes, globalization, packaging life cycles, as well as materials in contact with food, have increased the demand for strict safety measures in these fields

The link between food safety and health today is not only limited to the scientific field. Most consumers are highly sensitive to this issue, showing a more attentive attitude regarding ingredients and origin of food. In addition, the trend towards a healthy lifestyle has led producers to exploit the use of natural products. For example, due to the antioxidant properties some natural extracts are used in food field to delay oxidative processes of fat components; while others, due to anti-inflammatory and laxative properties, are used in the formulation of products (such as supplements and medical devices) useful to contrast gastro-intestinal disorders.

This PhD thesis is inserted in this complex scenario, observable from different points of view.

ABSTRACT

Chapter 1 deals with the development of a rapid analytical method for evaluating the distribution of molecular weights of polysaccharides present in commercially available formulations. The analyses were performed by size exclusion chromatography with refractive index detector (SEC / RI).

The development of the chromatographic method has been carried out on the basis of the possible structural characteristics of investigated polysaccharides that may affect the retentive process in SEC. After sample treatment methods optimization (final products and vegetable raw materials), the best typology of standards calibration of the chromatographic column was selected to estimate the molecular weight of analytes in real samples. Chromatographic evaluation of standards (PEO and dextran) and of the polysaccharide extracts was carried out using not only water as the eluent phase, but also buffer solutions at different pH and ionic strength. The proposed methods have been applied for a rapid analytical control of medical devices.

In *Chapter 2*, a study on the determination of acrylamide in potatoes is presented. Acrylamide was considered as a process marker to test new ovens with both "traditional" and "steam" cooking options. Cooking was carried out with "dry air circulation" function and replicated with "25% moisture" function, using different time and temperature conditions. Portions of cooked product characterized by different colors were sampled and analyzed to measure acrylamide levels, in order to create a colour scale indicating the different content of acrylamide. The purpose was to verify whether similar colours, even obtained under different cooking conditions, were characterized by similar acrylamide content. Analyses were performed by a liquid chromatograph coupled to a mass spectrometer, equipped with an electrospray (ESI) interface and a linear ion trap analyzer (LC-ESI-MS / MS). The content of acrylamide in crisp potatoes on the market has been also explored. In this case, the development of a new sample treatment to eliminate the fat component due to frying oil was necessary.

In *Chapter 3* a research about the development of appropriate preliminary sample preparation methods for characterization of nanomaterials contained into food is presented. Silver nanoparticles (AgNPs) are an example of nanomaterials that could be find in food due to their widespread use as antimicrobial, even if they are not allowed as food additive in European contest.

Initially, a literature study was conducted to evaluate methods to extract AgNPs from the media in which they were dispersed. From this evaluation it has emerged that one of the methods proposed is the Cloud Point Extraction (CPE). Since pH is one of the parameters influencing CPE, the stability and behavior of two types of AgNPs in media characterized by different pH

values has been evaluated. Comparison was made using Plasmon resonance (UV-VIS) and light scattering (DLS). In addition, to obtain information on shape and size distribution, suspensions of these nanoparticles in ultrapure water were analyzed by scanning electron microscopy in SEM mode (high vacuum conditions). The collected images were selected and analyzed using the ImageJ software.

In literature CPE is described for AgNPs, but not for these nanoparticles in complex matrices such as foodstuffs. For these reason a recent scientific article describing CPE as technique to separate AgNPs from environmental water was tested for its applicability to food matrices. AgNPs were evaluated in ultrapure water and in food matrices (juices and milk). Distribution of silver in the pellet and in the surfactant phases has been determined by ICP-MS after microwave digestion. In addition, different steps of CPE tested with AgNPs in ultrapure water have been examined by scanning electron microscope in different operation modes (Cryo-SEM, SEM and STEM), to confirm whether this extraction technique effectively preserve the shape and size of the nanoparticles.

Chapter 4 is based on the topic of Food Contact Materials. In particular, attention has been focused on a re-usable objects made of polycarbonate (PC) intended to come into contact with foodstuff. Migration tests on tableware of different age and degree of surface damage were performed, using ethanol and isooctane as food simulants (according to EU plastic regulation). Characterization and quantitative analysis of bisphenol A and some common additives used in plastic manufacturing were carried out by a new UHPLC-ESI-Orbitrap method, later validated.

Data were analyzed with the purpose of evaluating a possible correlation between bisphenol A and additives release, ageing, and surface integrity of plastic material.

Due to prolonged use over time, plastic objects inevitably undergo to degradation and may release unknown substances, called non-intentionally added substances (NIAS). For this reason, it was important to evaluate in simulants the presence of oligomers derived from a possible degradation of the material during ageing. Results obtained by UHPLC coupled to HR-MS were analyzed to explore a possible correlation between type and amount of different oligomers and age of the polycarbonate samples.

Migration experiments were also performed directly on food, to investigate in a complex matrix the possible presence of plastic additives, degradation products and colorants. Chocolate has been chosen as a model for a food with a high fat content and a new sample preparation methods was developed and validated. For the first time, HR-MS was applied for the identification and quantification of possible contaminants from food contact materials into chocolate.

Finally, in the section called *Collateral Activity*, the use of an Oxitest reactor to evaluate the oxidative stability of fat food over time, is presented. In particular, the stability of olive and extra-virgin olive oils enriched with natural extracts (essential oils and red chilly powders), olive oils stored in different packaging materials, tuna fish fillet in olive oil and biscuits with different flour mixtures was evaluated. Measures were performed by using a reactor that allows to perform in a fast (in terms of hours) and simple way (without fat removal from the food) accelerated oxidation tests, based on two parameters: temperature and oxygen pressure. At the end of each analysis, the induction period value (IP) is automatically generated: higher values correspond to longer oxidative stability of the product tested.

CHAPTER 1: SIZE EXCLUSION CHROMATOGRAPHY COUPLED TO REFRACTIVE INDEX DETECTOR (SEC-RI) FOR CHARACTERIZATION OF POLYSACCHARIDES FROM PLANT EXTRACTS

1. INTRODUCTION

1.1. Natural Polysaccharides

Natural polysaccharides from different plant sources are widely accepted as food source.

As well known, these complex compounds are considered as one of the major function components in food exhibiting multiple biological activities and health benefit effects. Moreover, plant-extracted polysaccharides have emerged as important bioactive compounds and have long been studied and widely used in different areas, such as medicine and pharmaceutics, owing to their biocompatibility, biodegradability, non-toxicity, and some specific therapeutic activities (Liu et al., 2015). The biological activities of polysaccharides are closely correlated to their physicochemical properties such as chemical composition, molar mass, extent of side chains/groups or substitution and their distribution on the backbone.

Polysaccharides possess a number of reactive functional groups in their chemical structure, including hydroxyl, amino, and carboxylic acid groups, indicating the possibility for chemical modification (Shukla & Tiwari, 2012). Molecular weight of naturally occurring polysaccharide varies between hundreds to thousands of Daltons, further increasing diversity.

With regard to the chemical composition, the polysaccharides are classified into two types, i.e. homo-polysaccharides or homoglycans, which are made up of a single type of monosaccharide, typical the example of cellulose and starch where both are polymeric carbohydrate consisting of a large number of glucose units joined by glycosidic bonds.; hetero-polysaccharides or heteroglycans, which consist of more than one type of monosaccharide, such as hemicelluloses, which include xylans, mannans, β-glucans with mixed linkages and xyloglucans. On other hand, to isolate the polysaccharides from the complex matrix is one of the most important tasks to deal with, and the characterization and quality control of polysaccharides is a challenge due the complexity of these compounds.

Water-soluble polysaccharides have attracted a great deal of attention because of their broad spectrum of therapeutic properties; they include starch and non-starch polysaccharides (NSPs).

Starch is a energy reserve in plants and is found mainly in legumes, cereals and tubers. It is present in the cytoplasm of vegetable cells as granules and consists of two molecules, α-amylose (about 20 %) and amylopectin (about 80 %). Amylose is composed of D-glucose units joined by α (1→4) glycosidic bonds. These long chains do not have branching and in water have an alpha helix conformation. Amylopectin chains have linear parts with α (1→4) glycosidic bonds, but every 25 - 30 D-glucose units there are branching points characterized by α (1→6) glycosidic bond. Amylopectins have a higher molecular weight distribution than α-amylose chains (from

1.000 to 500.000 Dalton) and can reach 100 million Dalton (Cabras & Martelli, 2004). Starch is the major carbohydrate reserve in higher plants. It provides nutrition for humans and animals, and is an important raw material in both food and non-food industries.

Non-starch polysaccharides (NSPs), instead, are the main structural components of the plant cell walls and form a major part of dietary fibre that are resistant to digestion and absorption in the human small intestine. Dietary fibers contain oligo and polysaccharides, as well as lignin and associated plant substances such as polyphenols and anthocyanins. Among dietary fibre polysaccharides: cellulose, hemicellulose, β-glucans, fructans, arabinoxylan, heteroxylans and pectins are mentioned the most often.

1.1.1. Polysaccharides and fibre

As above mentioned, the term *fibre* refers to a very heterogeneous class of compounds. The main constituents are non-starch polysaccharides (NSPs) found in plant cell walls where they are associated and / or replaced with proteins, other polysaccharides and phenolic compounds such as lignin (Cummings & Stephen, 2007).

The definition of dietary fibre can be considered from two points of view. Physiologically it is defined as "the dietary components resistant to degradation by mammalian enzymes", whereas chemically as "the sum of lignin and non-starch polysaccharides" that are not digested by endogenous secretion of the digestive tract of humans (Montagne et al., 2003; Rodriguez et al., 2006). In fact, digestive enzymes in the human body are able to break only α glycosidic bonds and for this reason NSPs fall into the chemical definition of fibre.

Fiber does not have a nutritional role but, thanks to the functional and metabolic effects of its components, is very important for the health of the human body. Based on interactions with water, the constituents of dietary fiber are classified in *soluble* and *insoluble*.

Soluble fibre increases the viscosity of the intestinal contents and is also fermented by the intestinal microbiota, producing short-chain fatty acids (SCFAs) which promote a number of beneficial health effects (Kumar et al., 2012). Soluble fibre components are oligo- and polysaccharides which are different for length and branching of the chains but also for the presence of different functional groups. Examples are fructans and fructo-oligosaccharides (FOS). The first are fructose polymers where momomeric units are joined throught β (2→1) glycosidic bonds. A glucose molecule, linked with α (1→2) glycosidic bond, can be found at the beginning of the chain. Their degree of polymerization (DP) can reach 60 units of fructose, such as inulin. FOS are glucose and fructose mixtures and have a DP from 3 to 7. Pectins are also part of the soluble fibre. These polysaccharides are made up of hundred units of D-galacturonic acid

esterified with methyl alcohol. Pectins are often associated with neutral polysaccharides in the plant cell walls, such as arabinans, galactans and arabinogalactans (McCann & Roberts, 1991).
Insoluble fibre, retaining water and gases present in the intestinal lumen, increases the stool mass and therefore speeds up its intestinal transit. Examples are cellulose, hemicellulose and lignin. Like starch, cellulose is also a homopolymer of D-glucose and monomeric units (about 3000) are joined throught β (1→4) glycosidic bonds. The type of chemical linkage makes this linear polymer non digestible for humans. Hemicellulose are branched heteropolysaccharides, where monosaccharides different from glucose are also present (such as xylans, galactans, mannans). Lignin instead is a three-dimensional aromatic polymer composed of phenylpropane derivatives and contributes to the structural rigidity of the plant cell walls, along with cellulose and emicellulose.

1.2. Physiological effects of NSPs

NSPs, the main constituents of dietary fibre have had a great importance over recent years in the biomedical field due to the positive effects on human health.
Viscosity, fermentability and water-holding capacity are characteristics of polysaccharides that affect their biological activity and depend on physical-chemical properties such as constituent monosaccharides, type of glycosidic bond between monomeric units and molecular size.
Polysaccharides that are part of the soluble fibre have an important prebiotic effect because, being well fermented by gut flora, they are able to selectively stimulate the growth and metabolic activity of bacteria important to regulate the wellness of the organism. Fermentative processes that occur in the colon lead primarily to the formation of SCFAs (in particular acetate, propionate and butyrate) which, by acidifying the intestinal environment, support the development of bacteria such as Bifidobacteria and Lactobacilli and prevent the growth of pathogenic species (Tungland, 2003). SCFAs are absorbed in the intestinal tract and used by tissues as an energy source. In particular, butyrate contributes to intestinal mucosal integrity, stimulating cell proliferation (Green, 2000; Sakata & Inagaki, 2001).
Interesting food ingredients in this respect are fructooligosaccharides (FOS) and inulin, water-soluble carbohydrates that are distinguished in fructans groups. Fructans are carbohydrate polymers consisting of a sucrose molecule that is elongated by a chain of fructosyl units connected through β-(2 → 1) or β-(2 → 6) linkages (1), depending on the linkage type they are called inulin and levan, respectively. Furthermore, it has also been demonstrated that FOS and inulins at different degree of polymerization shown different prebiotic capabilities (Corradini et al., 2004; Rossi et al., 2005; Sims et al., 2014).

Soluble polysaccharides have high water-holding capacity and in particular increase the viscosity of the chyme. This involves a delay in gastric emptying with a consequent increase in the sense of satiety and reduction in the rate of diffusion of substrates. In this way, glucose absorption is reduced and the glycemic excursion is lower. In addition, soluble polysaccharides also contribute to decreasing blood cholesterol levels (Gunness & Gidley, 2010).

Insoluble fibre polysaccharides, compared to the soluble counterpart, are resistant to digestive enzymes and have very low fermentability. For this reason and for their ability to retain water, they increase fecal mass and stimulate peristaltic intestinal action improving transit time. By accelerating evacuation, the contact times of the intestinal wall cells with potentially cytotoxic agents promoting carcinogenic events are reduced (Cummings et al., 1992).

Due to their positive physiological effects, fibre and polysaccharides, which are the most ubiquitous polymer found in nature, are very important for the wellness of the human body, and their application can be promoted in the food industry, as well as to cure human diseases.

Medical research allows today to find on the market numerous products added with these functional polysaccharides and individual fiber constituents can also be used for this purpose. For example non-starch polysaccharides extracted from leaves, flowers, roots of officinal plant species, are used to formulate useful products to contrast gastrointestinal problems, representing a valid alternative to the usual medicines employed.

As well known, bioactivities of polysaccharides are closely correlated to their molecular masses (Hu et al., 2013). Therefore, characterization of polysaccharides and their different fractions are critical in biomedical and functional food science. Up to date, many studies have been performed for the qualitative analysis of polysaccharides (Li et al., 2013).

The standard methods of analyzing polysaccharides include colorimetric assay, complete o partial hydrolysis, treatment with specific glycosidases (enzymatic cleavage), thin layer chromatography, capillary electrophoresis, HPLC an High-performance anion exchange chromatography coupled with pulsed electrochemical detection (HPAEC-PED), mass spectrometry, MALDI-TOF-MS and GC-MS (mainly for derivatized oligosaccharides), Size Exclusion Chromatography (SEC) coupled with refractive index detection or light scattering detection, as well as viscosity detectors (differential viscometers and single or dual capillary viscometers) (El Rassi, 1995), and the combination of SEC with Multi-Angle Laser Light Scattering detection, (SEC-MALLS) which has been demonstrated to be useful for identifying olygosaccharides, overcoming the limitations of column calibration (Wyatt, 1993; Han & Lim, 2004), Nuclear magnetic resonance spectroscopy (Tojo & Prado, 2003) and others.

Aim of this research work was the characterization of polysaccharides with a molecular weight greater than 20.000 Dalton, present in two commercially available products and used to contrast disorders such as gastroesophageal reflux and constipation. The analytical investigation has also been extended to vegetable extracts used in the formulation of these products. After determination of the experimental protocol for the extraction of the analytes of interest, their characterization was performed by size exclusion chromatography (SEC) using a refractive index detector (RID).

Size exclusion chromatography (SEC) is widely used for natural polysaccharides separation and characterization. SEC is a method where separation of different compounds occurs according the their size (hydrodinamic volume) measured by how efficiently they penetrate the pores of the stationary phase. This chromatographic technique was developed for the separation of large molecules, such as proteins, polymer, peptides, nucleic acid, starch, and polysaccharides, according to their molecular size when a solution is used to transport the sample through the pores of a stationary phase in a column. This technique was introduced in the 1950s as gel permeation chromatography (GPC) or gel filtration chromatography (GFC) (Kostanski et al., 2004).

In a pure size exclusion chromatographic separation, the separation mechanism is usually carried out solely on the size of the polymer molecules in solution, rather than any chemical interaction between particles and the stationary phase. Molecules of various sizes flow into the column and the smaller molecules flow more slowly through the column because they penetrate in the pores of the stationary used to pack the chromatographic column, whereas large molecules flow quickly through the column because they do not enter the pores. Consequently, polymers having a larger hydrodynamic volume elute from the column sooner than polymers having a smaller hydrodynamic volume. In gel permeation chromatography (GPC), a hydrophobic column packing material and a non-aqueous mobile phase (organic solvent) are used to measure the molecular weight distribution of synthetic polymers. The other is gel filtration chromatography (GFC), which uses a hydrophilic packing material and an aqueous mobile phase to separate, fractionate, or measure the molecular weight distribution of molecules soluble in water, such as polysaccharides and proteins.

Separations are usually carried out using commercially available HPLC columns packed with different packing material pore sizes (different exclusion limits).

Size exclusion chromatography is based on a relatively simple principle, but can involve complicated considerations when actually performing analyses or analyzing results. Selecting a suitable column and comparing the calibration curves are the main experimental operations to

find the range of molecule weights to be measured. Today, SEC is the most widely accepted and used analytical method for the measurement of the molecular weight distribution of polysaccharides. However, because of many new developments in instrumentation, there are many different configurations of SEC systems used.

This study has the purpose to optimize a fast method to determine the molecular weight distribution of polysaccharides by HPLC-SEC for industrial application.

2. MATERIALS AND METHODS

2.1. Chemicals

Ethanol, acetone (Chromasolv™) and sodium azide were purchased by Sigma- Aldrich (Milan, Italy). Sodium hydroxide, orthophosphoric, sodium nitrate and sodium citrate were obtained from Carlo Erba (Carlo Erba, Italy). Deionized water was obtained with a Milli-Q™ system (Millipore, Bedford, MA, USA). Standards of polyethylene oxide (PEO calibration kit) and dextrans from Agilent Technologies and Sigma- Aldrich (Milan, Italy) respectively, were used to calibrate the chromatographic column.

2.2. Samples

Two commercially available products used to contrast disorders of the gastrointestinal tract for their polysaccharides content were analysed. In particular, a gel product applied in the case of constipation and a tablet product used against gastrooesophageal reflux. The analytical investigation was also extended to vegetable extracts (*Aloe vera L.*, *Althaea officinalis L.*, *Matricaria Chamomilla L.*, *Malva sylvestris L.*, *Glycyrrhiza glabra L.*) and honey, used in the formulation of these medical device.

2.2.1. Samples treatment

Sample preparation was optimized considering methods related to the extraction process of polysaccharides from plant matrices (Cai et al., 2008; Renjie, 2008) and subsequently modified. The procedure applied was different depending on the physical state of the samples: solid in one case and gel in the other case.

1 g of gel sample was added to 7 ml of ethanol. Sample was stirred using vortex mixex for 30 seconds and stored overnight at 4 °C. Then it was centifuged at 8000 rpm for 20 minutes at 4 °C and supernatant obtained was eliminated. The precipitate was exposed to a nitrogen stream to remove residues of solvent and after it was added to 1 ml of distilled water. The sample was

stirred using vortex mixer for 30 seconds and incubated at 40 °C in a water bath for 1 hour. It was then centrifuged at 8000 rpm, 4 °C for 5 minutes and supernatant was filtered through a 0.2 μm disposable nylon filters. The addition of water to the pellet and the subsequent steps were repeated two more times and the filtered supernatants were collected (3 ml of final volume).

For tablet sample a slightly different protocol from the previous one was followed, especially at the initial stages. Two tablets were crushed using a mortar and pestle and 500 mg of powder were added to 7 mL of bidistilled water. Sample was kept under constant stirring in a water bath at 60 °C for 1 hour to facilitate the extraction process as much as possible. After centrifugation at 4 °C, 8000 rpm for 5 min, the extraction process was repeated two more times and the supernatants obtained were collected in a 25 ml flask. From here, 10 ml was taken, placed in a 50 ml plastic tube and then 30 ml of ethanol were added. After stirring using vortex mixer for 30 seconds, sample was stored overnight at 4 °C. At this point, the same steps for sample in gel were applied. Since most of the natural extracts (*Aloe vera L., Althaea officinalis L., Matricaria Chamomilla L., Malva sylvestris L., Glycyrrhiza glabra L*) employed in the formulation of the two products were in powder form, the protocol followed for tablet sample was extended to their treatment. Instead, the same extractive steps used for the gel product were used for honey from mixed blossom and honeydew.

2.3. Instrumental analytical method

2.3.1. SEC/RI analysis

The instrumental analysis of the samples was performed by size exclusion chromatography (SEC) coupled with a refractive index detector (RI). Chromatographic instrumentation was purchased from Agilent Technologies (Agilent Technologies Palo Alto, CA, USA) and equipped with Agilent 1200 isocratic pump, Agilent 1200 degasser, Agilent 1260 Infinity manual injector with a loop of 100 μL and RI detector Aglient 1260 Infinity. Chemstation for LC systems (Agilent Technologies) was used to control the instrument and for data collection.

In the first screening phase of the study, SEC separations were performed using a PL aquagel-OH MIXED-H 8μm 300 x 7.5 column (Agilent Technologies Palo Alto, CA, USA). This column with a wide range of molecular weights (6.000-10.000.000 Dalton) was calibrated employing polyethylene oxide standards (see Table 1). In the second phase of the study, to characterize analysts of interest, chromatographic separation were carry out using a PL aquagel-OH 40 8μm 300 x 7.5 mm column (Agilent Technologies Palo Alto, CA, USA). This column, characterized by a lower molecular weight range (10.000-200.000 Dalton) than the previous one, was calibrated

using polyethylene oxide (from 20.260 Dalton to 183.000 Dalton) and dextrans standards (see Table 2).

Table 1. Polyethylene oxide (PEO) standars used to calibrate the first SEC column

PEO molecular weight (Dalton)	Concentration (mg/mL)
20.260	50
30.310	100
49.640	50
71.800	50
134.300	50
183.500	50
298.000	50
498.600	25
692.000	25
905.000	25

Table 2. Dextran standard used to calibrate the second column.

Dextran molecular weight (Dalton)	Concentration (mg/mL)
12.000	1
25.000	
50.000	
80.000	
150.000	

All standard solutions were prepared by solubilization in bidistilled water under constant stirring (overnight for PEO with high molecular weight) and then filtered through a 0.2 μm disposable nylon filters. Stock solutions were kept away from light at 4 °C.

In general a mobile phase of bidistilled water with 0.02 % w/v sodium azide (NaN_3) was employed. To evaluate the effect that a buffered environment had on the analytes of interest, a mobile phase with sodium dihydrogen phosphate (NaH_2PO_4) and different pH values (pH 3, pH 7, pH 8) was used. The volume of the injected sample was 100 μL (with the injector completely filled).

Finally, the possible interaction of the analytes with the chromatographic support was considered: for this reason the samples were doped with sodium citrate and the effect of ionic strength on this

compound was evaluated, adding three different concentrations (0.2M, 0.4M, 0.8M) of sodium nitrate ($NaNO_3$) at mobile phases containing NaH_2PO_4 (pH 7.0 and pH 3.0).
All mobile phases were eluted with a flow rate 0.5 mL/min at room temperature.

3. RESULTS AND DISCUSSION

3.1. First step: screening analysis

During the first step of the research work, a screening analysis was carried out to establish approximately the molecular size of polysaccharides in the analyzed products. For this reason, samples were processed in order to extract the polysaccharides of interest. The experimental protocols used at this initial stage were not very different from the definitive ones described in *material and methods* section: they were different only for the presence of a washing step of the precipitate with ethanol and acetone to eliminate possible interfering substances.
There are several specific considerations one must make when developing a particular SEC method for the molecular weight characterization of polysaccharide of interest.
These include choice of column packing type, column packing particle size, column packing range of pore size, the mobile phase composition, temperature, molecular weight calibration method, and polymer concentration. In addition, to ensure high quality of the SEC analysis, there are also general considerations such as uniformity of the mobile phase flow rate during fractionation as well as a proper sample injection.
Considering that SEC separations are based on diffusion into and out the pores of the column's packing material, special care was paid in the selection of the column.
Chromatographic separation was performed using a PL aquagel-OH MIXED-H 8µm 300 x 7.5 mm column with a wide molecular range (from 6.000 to 1.000.000 Dalton).
High-Performance Liquid Chromatographic separation by SEC-IR are performed under isocratic conditions. However, some chromatographic parameters such as temperature, mobile phase composition and flow-rate were investigated to evaluate HPLC-SEC-IR separation.
As reported in the experimental section the mobile phase was bidistilled water with 0.02 % w/v sodium azide (NaN_3), eluting at a costant flow rate of 0.5 mL/min.
The reproducibility of the solvent flow rate is a factor of extreme importance in accurate qualitative, as well as, quantitative MW analyses and as reported in section 3.2.6 the effect of flow rate on the SEC separation of standard PEO and dextran was analysed.
In Figure 1 and 2, the chromatographic profiles of the gel product and the tablet product, respectively, are reported.

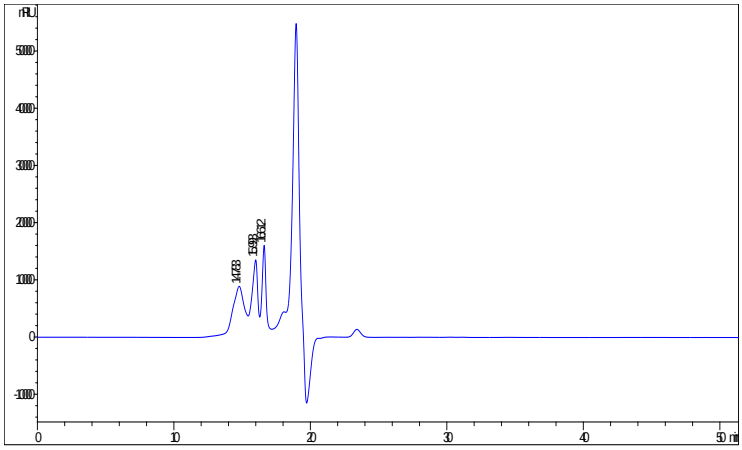

Figure 1. Chromatographic profiles of the gel product.

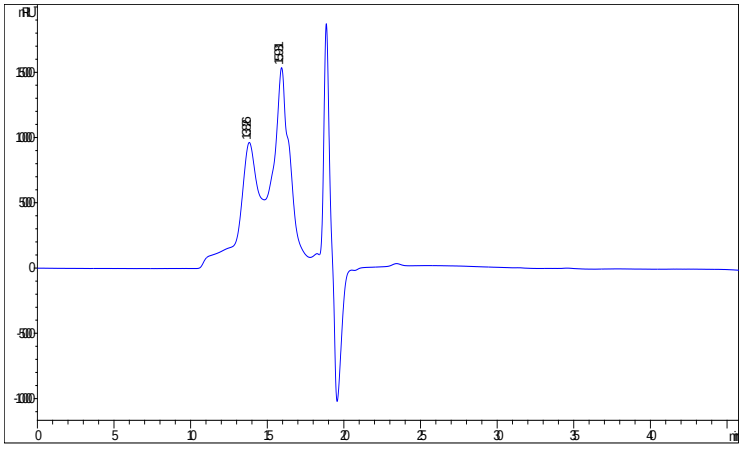

Figure 2. Chromatographic profiles of the tablet product..

In SEC, the size-based separation allows the construction of a calibration curve based on a set of known analytes, which can be used to estimate the molecular weight of unknown polysaccharides.

However, by SEC it is possible to provide molecular weight distribution and molecular weight averages for linear homo- and uniform composition copolymers. These are often referred to as simple polymers in the context of SEC. With simple polysaccharides and negligible band broadening, the SEC detector cell contains polymer chains having the same chemical composition and molecular weight. With complex polysaccharides (polymer chains nonuniform in composition and/or polysaccharides with a distribution of long-chain branching frequencies and branch lengths) and negligible band broadening, the contents of the SEC detector cell involve polymer chains with the same hydrodynamic volume but possibly with a wide range of compositions, molecular weights, and chain branching frequencies and lengths.

In this work, the column was calibrated by employing polyethylene oxide (PEO) standards, polymers obtained from a polymerization reaction in which the starting monomer is ethylene oxide. This reaction allows to obtain monodispersed polymers, thus avoiding the possibility of collateral reactions that could lead to a collapse of the average molecular weight of the polymer. This means that molecules obtained have all the same degree of polymerisation (DP) and the same size and for these reasons they are suitable to be used like a standards for Size Exclusion Chromatography.

The choice of these standards was based on their solubility in the aqueous mobile phase and on the opportunity to use monodispersed standards with high molecular weights in the range of interest.

According to the data sheets and to avoid a broadening of the chromatographic peak (overloading) which may occur when the viscosity of the polymer solution is too high, calibrating solutions were prepared at different concentrations depending on the molecular weight of the PEO standards (see Table 3). Chromatographic separation were performed with an operating flow of 0.5 ml / min. In this condition all PEO standards with molecular weight from 20.000 Dalton to 905.000 Dalton were eluted in about 3 minutes. Figure 3 shows the chromatographic profiles of greater and lower molecular weight PEO standards.

Table 3. Standard PEO employed

Molecular Weight (Dalton)	Concentration (mg/mL)	Retention time (min)
20.260	50	15.99
30.310	100	15.68
49.640	50	15.27
71.800	50	14.96
134.300	50	14.52
183.500	50	14.29
298.000	50	13.84
498.600	25	13.42
692.000	25	13.20
905.000	25	13.10

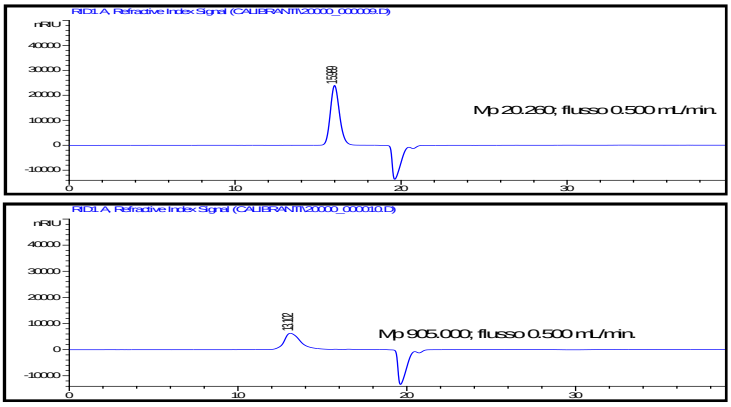

Figure 3. Chromatographic profiles of greater (905,000 Dalton) and lower (20260 Dalton) molecular weight PEO standard.

Comparing the retention times between the PEO standards and the peaks in the chromatographic profiles of the two products, it was possible to estimate the molecular weight range of polysaccharides characterizing the gel product (in the range from 20.000 to 180.000 Dalton) and the tablet product (20.000 – 450.000 Dalton). All this was confirmed employing a calibration curve (see Figure 4) obtained by plotting the retention time of the polyethylene oxide standards (x-axis) against the logarithm of their molecular weight (y-axis).

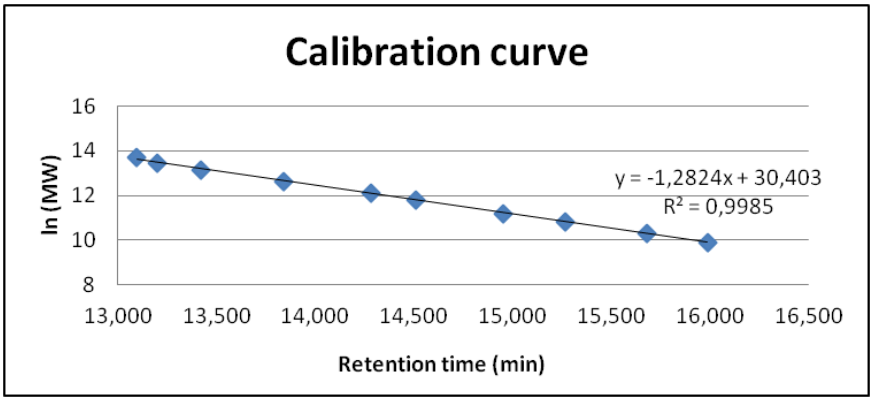

Figure 4. Calibration curve for PL aquagel-OH MIXED-H 8µm 300 x 7.5 mm column

3.2. Second step: characterization of the analytes of interest.

In this second step of the research program, more chromatographic columns were tested, in order to get a more accurate identification of the molecular weight of the analytes. Table 4 shows the columns considered at this stage.

Table 4. SEC column evaluated in the second step.

SEC column	Molecular range (Dalton)
PL aquagel – OH 50 8µm 300 x 7.5 mm	50.000 – 600.000
PL aquagel – OH 40 8µm 300 x 7.5 mm	10.000 – 200.000

The two columns in series allowed a window of about 9 minutes for elution of PEO standards with a molecular weight between 20.000 Dalton (retention time of 29.8 min) and 498.000 Dalton (retention time of 21.0 min). Although this would have led to a more accurate determination of molecular weights, the time and cost of analysis would be remarkable. For this reason it was chosen to use only PL aquagel - OH 40 8µm 300 x 7.5 mm column, which had a range of molecular weights in accordance with the aim of the research (characterization of polysaccharides with a molecular weight greater than 20.000 Dalton). Figure 5 shows the chromatographic profiles of PEO standards with 20.260 and 183.000 Dalton molecular weight used to define the time window of interest.

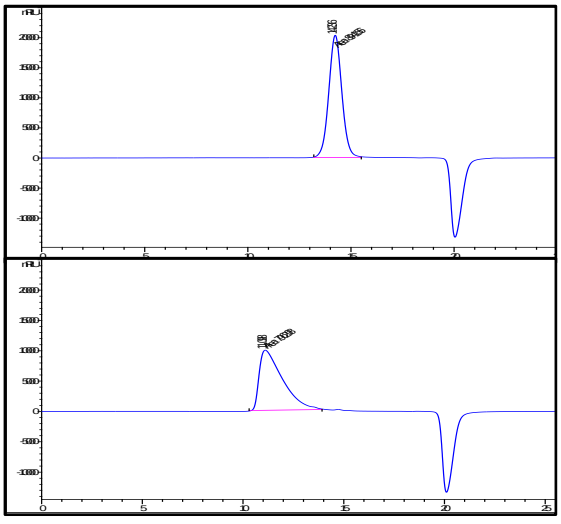

Figure 5. Chromatographic profiles of PEO standard 20.260 Dalton and 183.000 Dalton obtained using PL aquagel – OH 40 8µm 300 x 7.5 mm column.

3.2.1. Qualitative analysis

3.2.2. Definition of extraction procedures

In order to assess whether the analytical procedures used to extract the analytes of interest were correct and exhaustive, extraction yields were considered. This was done only on the gel product and the tablet product, because they were more complex than the natural ingredients employed in their production.

Polysaccharides are not soluble in alcohol thus, thanks to a phase of the extraction procedure involving the use of ethanol, it was possible to isolate them from the matrix in a pellet formed after centrifugation. For this reason PEO standards, which are soluble both in water and in ethanol, were not employed to fortify the real samples. The compounds suitable for this purpose were dextrans (branched glucose polymers synthesized by the polymerization of sucrose molecules) which, like target analytes, were water-soluble but insoluble in ethanol.

A dextran with a retention time greater than standard PEO 20.260 Dalton was used, because it eluted out of the area of interest. For this reason samples were fortified employing a dextran with a molecular weight of 12.000 Dalton.

Three experimental protocols to determine polysaccharide extraction procedures from the samples were evaluated. The procedures were very similar to each other and were different only for phases after the step overnight at 4 °C.

In experiment 1 after centrifugation (8000 rpm, 4 °C for 20 minutes) and removal of the supernatant, the precipitate was added to 1 ml of ethanol (twice), stirred using vortex mixer for 30 seconds and centrifuged (8000 rpm, 4 °C for 5 minutes). These same steps were repeated with 1 ml of acetone (twice).

In experiment 2 after centrifugation (8000 rpm, 4 °C for 20 minutes) and removal of the supernatant, washings of the precipitate with ethanol and acetone were not performed.

In experiment 3, after centrifugation (8000 rpm, 4 °C for 20 minutes), the pellet was stored in refrigerator, while the supernatant was placed in a 50 ml falcon tube and added with 20 ml of ethanol. The sample was stirred using vortex mixer for 30 seconds and left overnight at 4 °C. After centrifugation (8000 rpm, 4 °C for 20 minutes), the supernatant was discarded and the pellet was dried with a stream of nitrogen to remove traces of solvent. The precipitate was added to 1 ml of bidistilled water, stirred using vortex mixer for 30 seconds, transferred over the first pellet obtained.

In all three tests, addition of 1 ml of water to the precipitate dried in nitrogen stream, incubation in a water bath at 40 °C for 1 hour, centrifugation at 8000 rpm, 4 °C for 5 minutes and filtration of the supernatant with 0.2 μm disposable nylon filters, was repeated three times. Table 5 shows tests performed and the extraction yields obtained.

Table 5. Tests performed for tablet product and gel product. Extraction yields are expressed as areas ratio.

Test performed	Tablet product (extraction yield %)	Gel product (extraction yield %)
Test 1 (washing of the precipitate)	90,21	115,55
Test 2 (no washing of the precipitate)	99,74	111,25
Test 3 (no washing of the precipitate and double precipitation of the supernatant)	83,61	123,82

In the case of the tablet product (see Table 5), the highest extraction yield was obtained by eliminating precipitate washing with acetone and ethanol. Test 1 provided a slightly lower percentage yield, but required more solvents and longer working times. Likewise, test 3 had the lowest extraction yield and provided excessive sample preparation times. Thus, test 2 was selected as a definitive protocol for the treatment of the tablet product.

In the case of the gel product (see Table 5), the highest extraction yield was obtained by eliminating pellet washing and the double precipitation of the supernatant. The small differences in the extraction yields obtained in the three tests led to the choice of test 2 like a definitive protocol for the treatment of the gel sample. In Figure 6 and 7, there are the chromatographic profiles for the two samples (gel and tablet), obtained with the final extraction procedure.

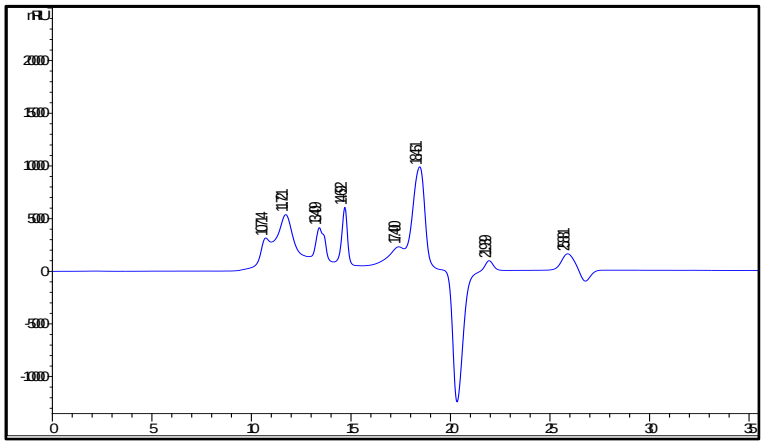

Figure 6. Chromatographic profile of the gel product.

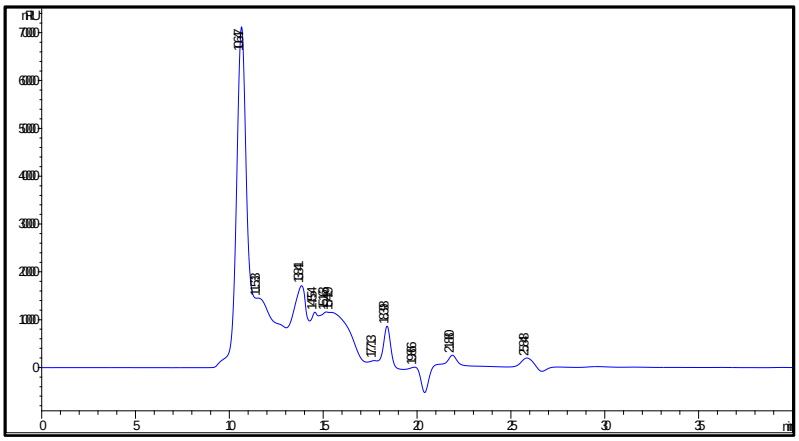

Figure 7. Chromatographic profile of the tablet product.

After the definition of the extraction method for the polysaccharide fraction from the two products, the protocol was also applied to the natural ingredients used in their formulation. Since most of the natural extracts (*Aloe vera L.*, *Althaea officinalis L.*, *Matricaria Chamomilla L.*, *Malva sylvestris L.*, *Glycyrrhiza glabra L*) were in form of powder, the protocol followed for the tablet product was extended to their treatment. Instead, the same extractive steps employed for the gel product were used for multi-flower honey and honeydew.

The chromatographic profiles obtained for the natural extracts are reported below.

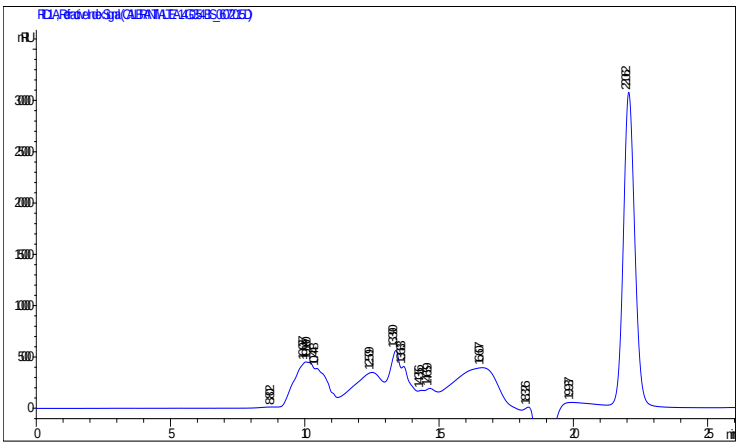

Figure 8. Chromatographic profile of *Althaea officinalis L.*

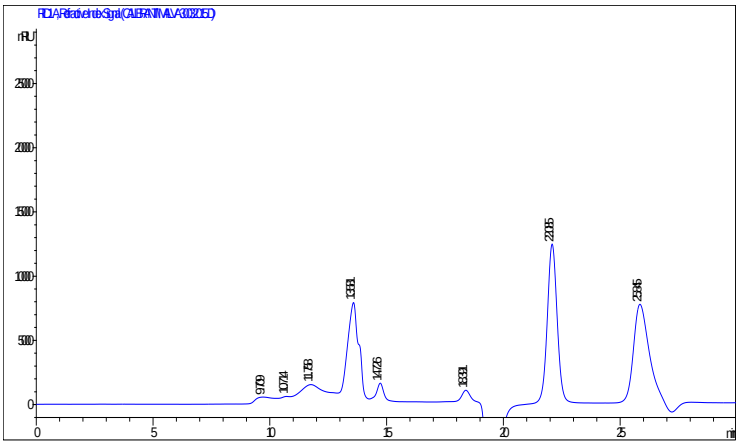

Figure 9. Chromatographic profile of *Malva sylvestris L.*

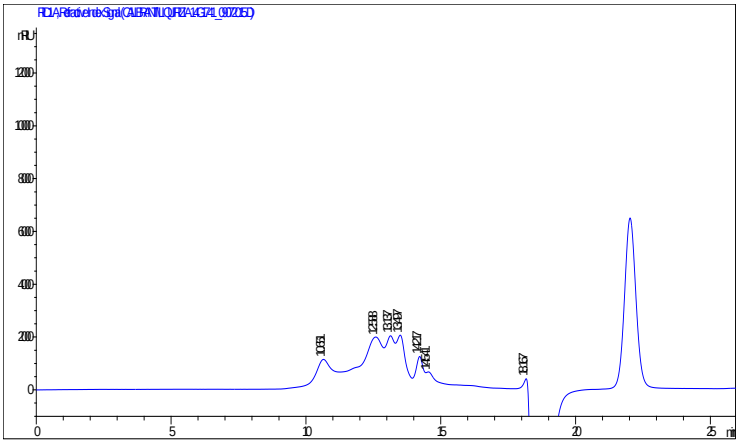

Figure 10. Chromatographic profile of *Glycyrrhiza galbra L.*

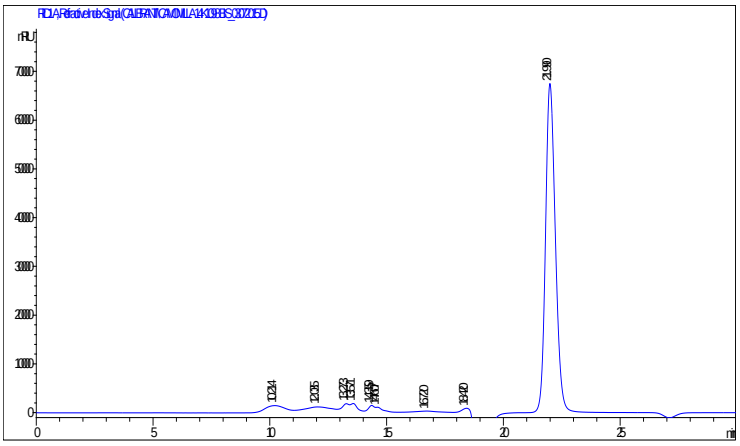

Figure 11. Chromatographic profile of *M. Chamomilla L.*

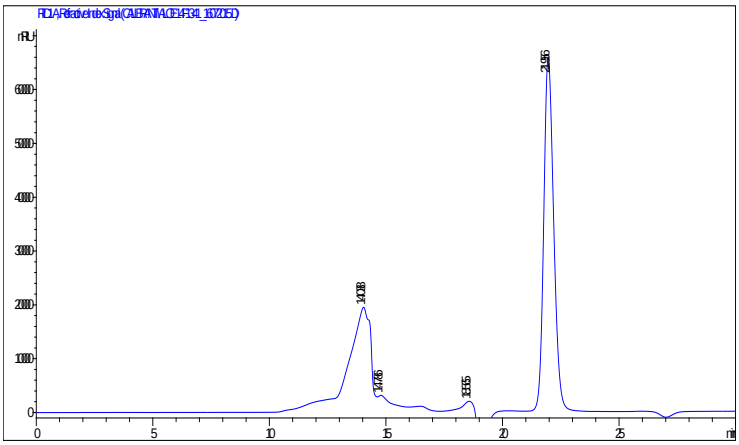

Figure 12. Chromatographic profile of *Aloe vera L.*

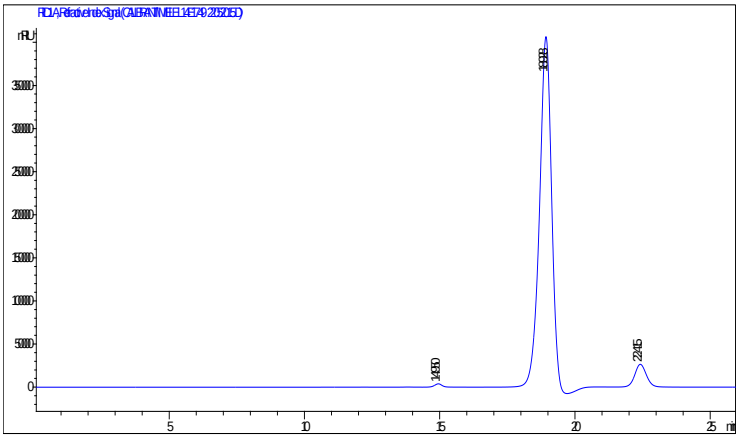

Figure 13. Chromatographic profile of Multi-flower honey

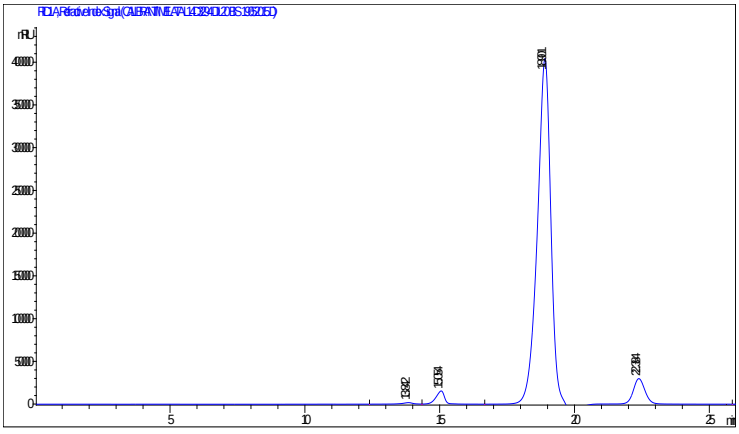

Figure 14. Chromatographic profile of honeydew

As can be seen from the *Althaea officinalis L.*, *Malva sylvestris L.*, *Glycyrrhiza galbra L.* and *M. Chamomilla L.* chromatographic profiles, a homogeneous distribution of polysaccharides with a molecular weight of between 20.000 and 200.000 Dalton was reported. *Aloe vera L.* instead had the most polysaccharides with molecular weight around 20.000 Dalton.

For the two types of Honey, the peaks found may be due to polysaccharides with a low molecular weight (less than or equal to 20.000 Dalton) not present in the raw material but resulting from enzymatic transformations due to glandular secretions of the bees and leading to the formation of disaccharides, trisaccharides and higher sugars (Jan Mei et al., 2010; Corradini et al., 2012).

3.2.3. SEC column calibration: PEO standards versus dextran standards.

As already mentioned, the calibration curve is important to determine approximately the molecular weights of polysaccharides present in the analyzed samples. The calibration of the column selected in this second step was initially made employing PEO standards, but after the evaluation of data present in literature (Reynolds & Dweck, 1999) it was decided to use dextran standards and compare their curves (Figure 15). For the two types of standards, molecular weights covering the molecular range of the selected column (10.000 to 200.000 Dalton) were chosen.

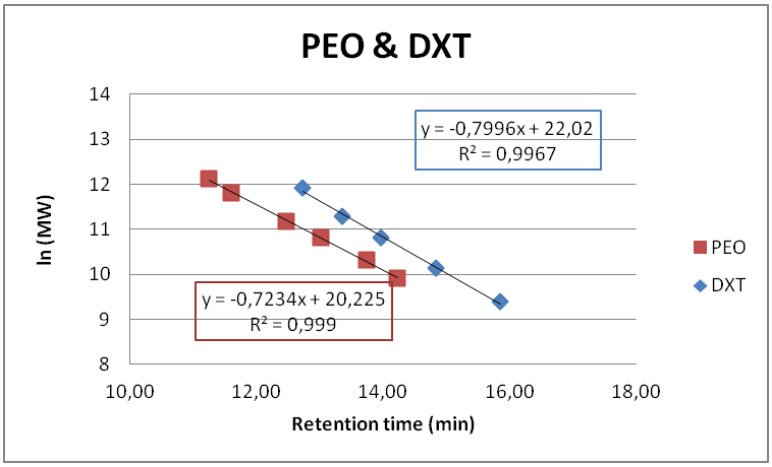

Figure 15. Comparison between polyethylene oxide (PEO) calibration curve and dextran calibration curve (DXT).

As shown in Figure 15, both calibration curves had a linear trend, but standards with similar molecular weight show different retention times (Dextran 50.000 Dalton, retention time 13,96 min; PEO 49.640 Dalton, retention time 13,02 min).

This finding demonstrates that size exclusion chromatography is not based only on the molecular weight, but on the real molecular dimension of the analytes (Agilent Technologies, 2015). Also, they may have different conformations on the basis of the solvent / buffer used like mobile phase.

The mobile phase employed in this research work was only from MilliQ water containing a small percentage (0.02 %) of sodium azide to avoid algae formation; it could therefore be hypothesized that, apart from the different nature of the two standards, the different retention time could be due to a different thickness of the solvation shell around the two molecules.

After evaluations on these two types of standards, it was decided to use the dextrans to calibrate SEC column because, compared to PEO, they have a natural origin and from a molecular point of view they are much more similar to polysaccharides.

3.2.4. Influence of mobile phase composition

Although separations were performed using as the mobile phase bidistilled water containing 0.02 % (w/v) sodium azide (NaN_3), to evaluate the presence of mixed mode interactions between analytes and the stationary phase, some parameters in mobile phase composition were investigated.

3.2.4.1. Mobile phase pH

Considering that the influence of pH on both secondary interactions and the structure of the complex polysaccharides present in the analysed samples, the effect of mobile phase pH was investigated using as eluent mobile phases prepared dissolving in water sodium dihydrogen phosphate (NaH_2PO_4) and adjusting the pH at three different values (pH 3, pH 7, pH 8). These analyses were performed to evaluate the effect of pH on the column calibration. The volume of the injected sample was 100 µL (with the injector completely filled).

The two standards considered for calibration of the chromatographic column are very different (see Figures 16 and 17). PEO standards are more hydrophobic than dextrans and this influences the possible interaction between standards and the chromatographic support employed, which is a polymer having hydroxyl groups. Thus, the hydrophilic molecules (dextran standards) interact more with the stationary phase and this is confirmed by the fact that the dextran 25.000 Dalton elutes after PEO 20.260 Dalton (see Figure 18).

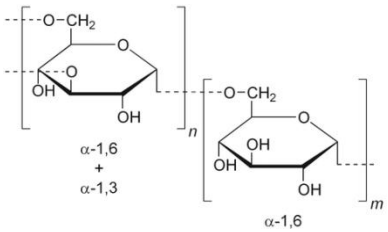

Figure 16. Dextran structure

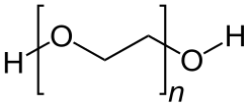

Figure 17. PEO structure

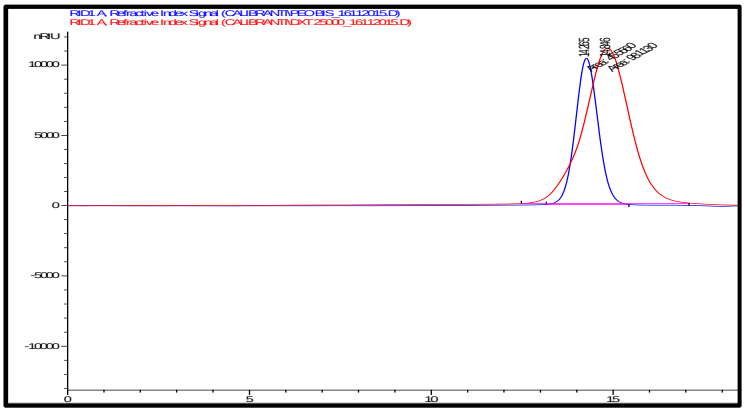

Figure 18. Chromatographic profiles of 20.260 Dalton (blue) and dextran 25.000 Dalton

Initially both PEO and dextran standards calibration curves were constructed to evaluate the possible influence of mobile phase-pH on the retention time of the standards (see Figures 19, 20 and 21). Standard solutions were prepared in water at a concentration of 1 mg/ml. The instrumental analysis were performed in duplicate.

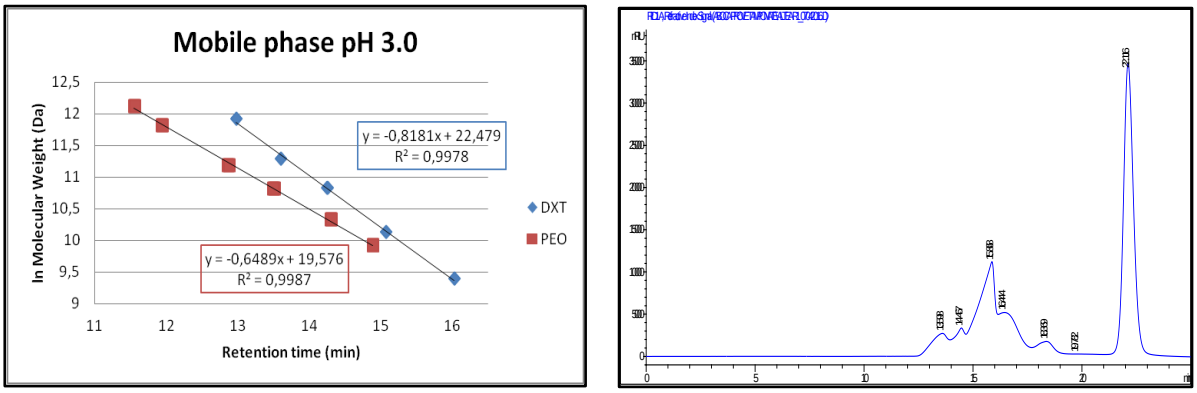

Figure 19. Calibration curves (left) for PEO and dextran standards obtained with a mobile phase at pH 3.0. Chromatographic profiles of *Althaea officinalis L.* (right) obtained employing mobile phase at pH 3.0.

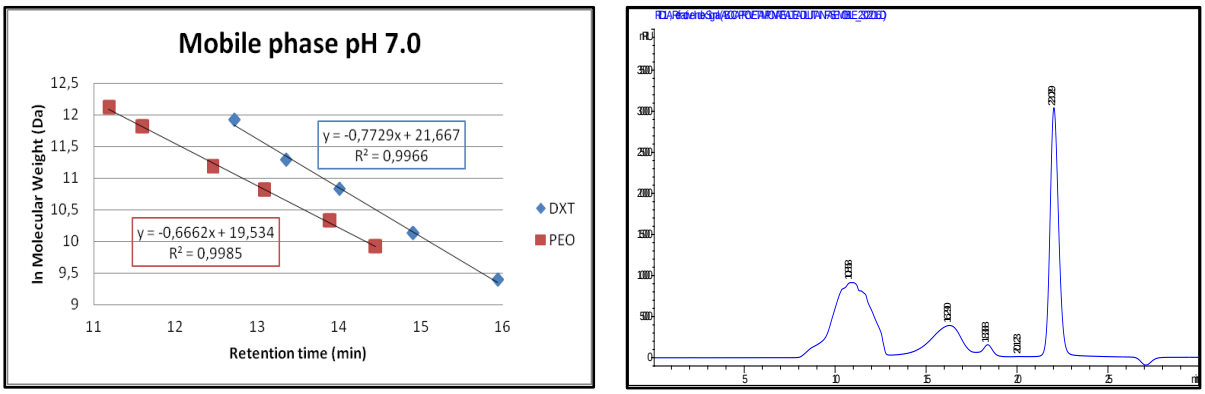

Figure 20. Calibration curves (left) for PEO and dextran standards obtained with a mobile phase at pH 7.0. Chromatographic profiles of *Althaea officinalis L.* (right) obtained employing a mobile phase at pH 7.0.

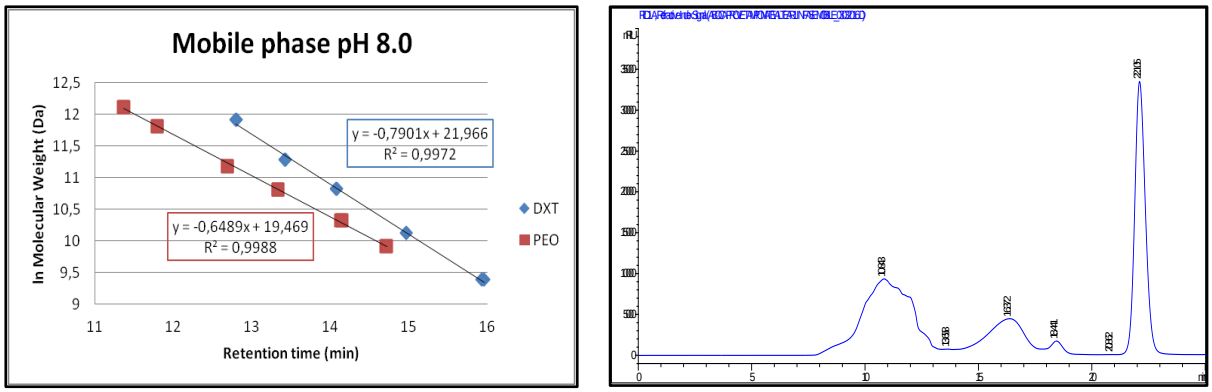

Figure 21. Calibration curves (left) for PEO and dextran standards obtained with a mobile phase at pH 8.0. Chromatographic profiles of *Althaea officinalis L.* (right) obtained employing a mobile phase at pH 8.0.

As can be seen from the calibration curves above, the use of eluent at different pH value did not show any effect on the two types of standards, suggesting pH has no effect on calibration under the conditions tested.

In real samples, however, it was possible to notice an improvement in the resolution of the chromatographic peaks (see Figures 19, 20 and 21). This could be explained by the fact that polysaccharides present in the vegetable world are a very heterogeneous class (Reynolds & Dweck, 1999). Depending on their structure and on the basis of the joined molecules (such as organic acids), they may have a more or less hydrophilic / hydrophobic character that also depends on the degree of protonation / deprotonation of the carboxyl and hydroxyl groups present.

3.2.4.2. Ionic strenght effect

The possible interaction of the analytes with the chromatographic support was evaluated, adding three different concentrations (0.2M, 0.4M, 0.8M) of $NaNO_3$ at mobile phases containing NaH_2PO_4 adjusted at pH 7.0 and 3.0.

Theoretically by increasing the concentration of $NaNO_3$, possible charges on the chromatographic support should be neutralized and therefore should not have an ionic interaction with the stationary phase but only a molecular sieve effect.

In SEC-IR, different structural features of polymers, such as branching, topology, composition, functionality, can affect the separation mechanism. All of them, could contribute to change from exclusion phenomenon to attractive interaction and adsorption chromatography or some undesired mixed-mode separations (Berek, 2010).

The chromatographic columns employed in our study show high resistance against interaction with neutral, multiply-charged, polyanionic and polycationic samples.

Comparing results as above reported, it can be concluded that generally, at the optimum conditions, the interaction of non ionic and polar analytes as PEO and Dextrans with the column matrix is rather weak due to the hydrophilic polyhydroxy functions on the surface of the column matrix.

However, much more attention could be payed in plant polysaccharide separation, which are usually constituted by a complex mixture of carbohydrates having different characteristics from each other.

The complexity of the polysaccharides present in the various ingredients that make up the tested commercial products would require a thorough study.

As a possible example of different chromatographic behaviour found in the polysaccharide fraction present in natural plant extracts, section 3.2.5 shows the study of the chromatographic profile of the polysaccharide fraction of an Althaea extract.

3.2.5. Study of SEC behaviour of one of the ingredient present in the investigated products

This part of the investigation was focused to characterize polysaccharide content in *Althaea officinalis L.*, which is one of the components present in the tablet product indicated for the treatment of problems related to acidity, such as burning, pain, gastroesophageal reflux and gastritis.

Althaea officinalis L. is a popular herb from the Malvaceae family, is a perennial plant native throughout salty marshes or wet uncultivated ground in southern Europe and now is cultivated throughout the world. It has been known from ancient time as a medicinal plant. *Althaea officinalis L.* contain about 5–11 % of water-soluble polysaccharides, mainly galacturorhamnans, arabinans, glucans and arabinogalactans (Deters et al., 2010; Sarikanat et al., 2014; Pakrokh Ghavi, 2015), and it has been found that the main components responsible for therapeutic effect of *Althaea officinalis L.* is the acidic heteropolysaccharide rhamnogalacturonan. (Šutovská et al., 2009).

Oral application of althaea extracts is related to the bioadhesive effects of the polysaccharides to the epithelial mucosa, protecting the irritated cells from local irritation (Deters et al., 2010).

Our study was focused on the characterization of the polysaccharide fraction obtained by extraction as reported in the experimental section.

Figure 22 reports the chromatographic profile obtained by eluting the sample with pure water.

The chromatographic profile highlights the presence of eight distinct peaks, where the peak at 10.75 minutes indicates the polysaccharide's fraction having a molecular weight greater than or equal to that of the exclusion of the chromatographic column.

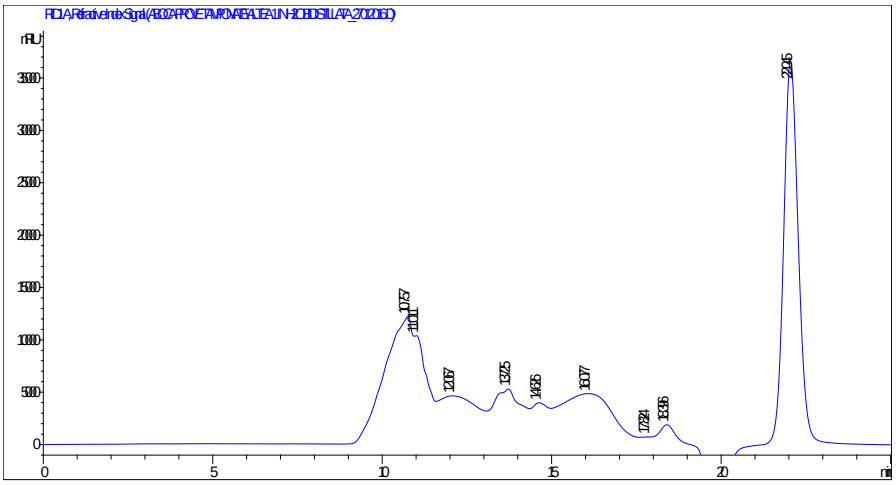

Figure 22. Chromatographic profile of *Althaea officinalis L.* obtained employing pure water as mobile phase.

As reported in Figure 23 increasing pH value of the mobile phase, a lower selectivity is obtained and the not excluded polysaccharides have slightly higher exclusion times. Moreover, at time 10.84 the eluted peak appears as the co-elution product of several chromatographic peaks (at least three) showing at this pH value greater selectivity towards higher average molecular weight polysaccharides.

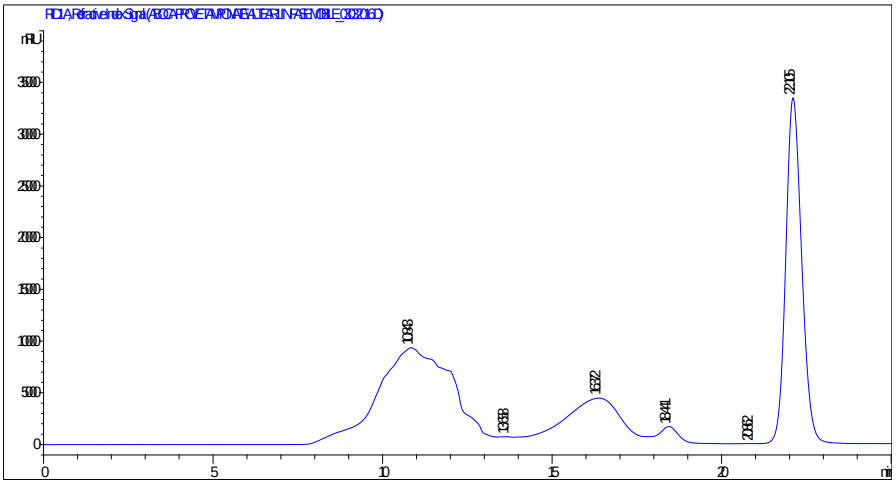

Figure 23. Chromatographic profile of *Althaea officinalis L.* obtained employing a mobile phases at pH 8.0

These results could be demonstrate that the retention volume these polysaccharides results from a combination of two effects: size-exclusion and ion-exclusion.

3.2.6. Flow rate

Resolution in size based separations can be influenced by linear velocity. Although using lower flow rates results in longer run times, the increased resolution gives greater confidence in aggregate quantitation.

In order to test the reliability and robustness of the method, the effect of flow rate on the SEC were analyzed at flow rates of 0.3 and 0.5 mL/min. Analysis of the separations shows no significant change in resolution with flow rate. However, decreasing the flow rate did not increase the resolution of the tested polysaccharides. A flow rate of 0.5 mL flow rates allow for greater throughput and faster analyses times.

3.2.7. Quantitative analysis

Also for the quantification of polysaccharides with molecular weight greater than 20.000 Dalton a comparison between PEO and dextran standards was made. Two calibration curves were obtained in the range of 10-500 μg ml^{-1}, for PEO 20.260 Dalton standard and for 25.000 Dalton dextran respectively. Standard solutions were prepared in water and for each concentration level instrumental analysis were made in triplicate. Below there are the calibration curves obtained for the two standards employed (see Figures 24 and 25).

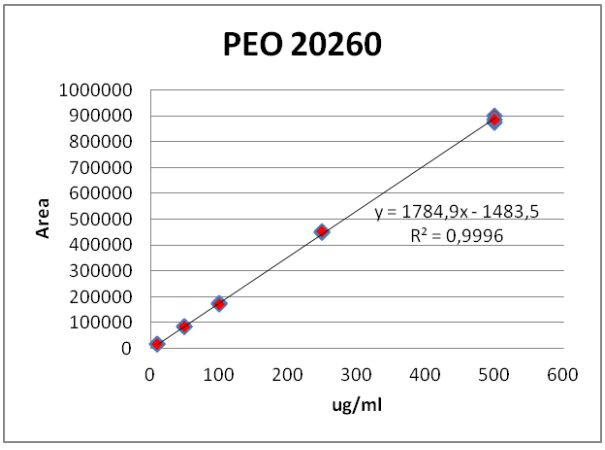

Figure 24. PEO calibration curve

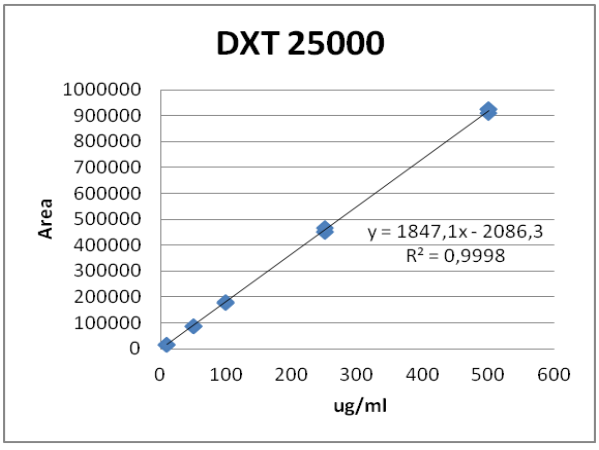

Figure 25. Dextran calibration curve

Table 6 shows the average amount of polysaccharides (expressed in g/100g and with standard deviation) for two commercially available products (gel and tablet).

Table 6. Average amount of polysaccharides for the gel product and for the tablet product.

POLYSACCHARIDES > 20.000 Da (g/100 g)	**Tablet product**		**Gel product**	
	PEO 20.260 Da	**DXT 25.000Da**	**PEO 20.260 Da**	**DXT 25.000 Da**
	1.952 ± 0.071	2.038 ± 0.050	0.140 ± 0.008	0.180 ±0.008

As can be seen in Table 6, using the calibration curve obtained with dextran standard 25.000 Dalton, there was an increase of 4.4% for the tablet product and 28.5% for the gel product.

This was a further confirmation that dextrans were the appropriate standards for the aim of this research work.

Both for the gel product and for the tablet product, different production line batches were analyzed (see Tables 7 and 8).

Table 7. Polysaccharides amount for the tablet product in different batches.

POLYSACCHARIDES > 20.000 Da (g/100 g)	**Tablet product**	
	PEO 20.260 Da	**DXT 25.000Da**
Batch A	1.915 ± 0.011	1.998 ± 0.009
Batch B	1.929 ± 0.010	2.039 ± 0.006
Batch C	1.907 ± 0.014	2.007 ± 0.006
Batch D	2.057 ± 0.006	2.109 ± 0.007

Table 8. Polysaccharides amount for the gel product in different batches.

POLYSACCHARIDES > 20.000 Da (g/100 g)	**Gel product**	
	PEO 20.260 Da	**DXT 25.000Da**
Batch A	0.144 ± 0.001	0.182 ±0.001
Batch B	0.129 ± 0.000	0.184 ± 0.001
Batch C	0.151 ± 0.001	0.191 ± 0.001
Batch D	0.138 ± 0.007	0.166 ± 0.009
Batch E	0.147 ± 0.001	0.182 ± 0.009
Batch F	0.138 ± 0.002	0.174 ± 0.001
Batch G	0.132 ± 0.001	0.179 ± 0.002

Small differences in amount of polysaccharides present in different production batches are due to the raw materials employed and depend, for example, on the collection period, plant maturity, geographic origin and type of storage.

4. CONCLUSIONS

More of the polysaccharides present in the analyzed samples are heterogeneous and branched polysaccharides including glucomannans as well as acemannan which is composed of a long chain of acetylated mannose and considered the main polysaccharide present in Aloa vera extracts. On the other hand, the aim of this work was the development of a rapid SEC-HPLC method which can be extensively used to separate polysaccharides mainly on the basis their size. For this reason, after evaluations on the two types of standards, it was decided to use the dextrans to calibrate SEC column because, compared to PEO, they have a natural origin and from a molecular point of view they are much more similar to the main polysaccharides present in analyzed extracts.

By considering the influence of mobil phase composition, the use of eluent at different pH value did not show any effect on the two types of standards. In real samples, however, it was possible to notice an improvement in the resolution of the chromatographic peaks. Depending on their structure, and on the basis of the joined molecules (such as organic acids), polysaccharides may have a more or less hydrophilic / hydrophobic character that also depends on the degree of protonation / deprotonation of the carboxyl and hydroxyl groups present.

Finally, by increasing ionic strenght of the mobile phase possible charges on the chromatographic support should be neutralized and therefore should not have an ionic interaction with the

stationary phase but only a molecular sieve effect. At the optimum conditions, the interaction of non ionic (PEO) and polar analytes (Dextrans) with the column matrix is rather weak due to the hydrophilic polyhydroxy functions on the surface of the column matrix. However, much more attention could be payed in plant polysaccharide separation, which are usually constituted by a complex mixture of carbohydrates having different characteristics from each other.

The results from this technique are comparable with others reported in literature. The HPLC-SEC-IR proposed method represents a rapid alternative to standard methods.
The industrial applications of this technique might be for quality control, study of fractionation processes and determination of purity.

5. REFERENCES

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CHAPTER 2: ACRYLAMIDE DETERMINATION IN POTATOES BY HPLC-MS AND CORRELATION WITH COLOUR INDICES

1. INTRODUCTION

1.1. Acrylamide

Acrylamide is a neurotoxin and a probable human carcinogen (Schulze & Siegers 2004; Taeymas et al., 2005). Although the toxicity of this substance is known, its toxicological effects are still poorly understood and controversial. Studies on cell cultures and laboratory animals have highlighted that acrylamide exposure can induce genetic modification, cancer as well as neurological damages and low fertility (Friedman, 2003; Sharp, 2003; Dybing & Sanner, 2005).

The first evidence of acrylamide in foods was highlighted by the Swedish National Food Administration. In April 2002, Swedish researchers published a report about acrylamide levels in potato products and oven-baked foods (Kepekei Tekkeli et al., 2012). The world alarm was immediate, since the levels of acrylamide in the evaluated foods were up to 500 times the levels allowed by the World Health Organization for drinking water (Lofstedt, 2003).

After the discovery of acrylamide in food, several research projects have been launched in Europe. For example, the HEATOX project (Heat-generated food toxicants: Identification, Characterisation and Risk Minimisation) was funded by the European Commission from 2003 to 2007. The aim was to estimate the health risks arising from the consumption of foods that could develop dangerous substances after heat treatment. The project has been focused on acrylamide, but other hazardous compounds such as furan and hydroxymethylfurfural (HMF) have been found in heat treated foods. Thus, to minimise their occurrence, mechanisms of formation, raw material composition, cooking and processing methods and inhibiting factors have been studied. Results obtained in the areas of formation, analysis, hazard characterization, risk assessment and characterization were used in publications, workshops and conferences. The HEATOX project involved 24 partners in 14 countries and it was linked with other research activities such as COST 927 Action. The objective of this action entitled “Thermally Processed Foods: Possible Health Implications” was to improve the quality and safety of thermally treated foods, considering the heat-induced contaminants (such as mutagenic heterocyclic amines and acrylamide) and the formation of health-promoting components (such as antioxidants and antimicrobial agents). The action lasted from 2004 to 2009 and involved 28 countries organized in 5 working groups: (1) analytical methods, formation pathways, EU regulation; (2) biological methods, risk assessment, consumer perception; (3) process optimisation and new developments; (4) absorption and physiological effects; (5) in vivo transformations and maintaining health. Results achieved were published in several scientific journals.

Interest about acrylamide has increased in recent years. Researches have shown that the main cause of acrylamide accumulation in the human body is due to specific foodstuffs (Tereke et al.,

2002) such as cakes, bread and fried or baked potatoes, but also in non-starch foods such as black olives and coffee (Casado & Montano 2008; Casado et al., 2010; Casado et al., 2013; Casado et al., 2014).

On the other hand, until now, acrylamide levels have not been found in boiled or steamed foods.

The discovery of high concentrations of acrylamide in foods has raised many concerns. In 2007 the European Commission adopted a Recommendation (EC) 2007/311 on the monitoring of acrylamide levels in food (COMMISSION RECOMMENDATION of 3 May 2007) which was extended in 2010 by Commission Recommendation (EU) 2010/307 (COMMISSION RECOMMENDATION of 2 June 2010). The aim was to better investigate the dietary acrylamide intake (with high attention to infants and baby foods) and to identify possible strategies for reducing this substance in food products.

In November 2017 the European Commission has adopted the Regulation (EU) 2017/2158 (COMMISSION REGULATION of 20 November 2017) which establishes the mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food. This regulation is addressed to food business operators which produce and place on the market foodstuffs. The effectiveness of mitigation measures must be verified by the food sector operators, through sampling and analysis as required by Article 4. Furthermore, in case the benchmark levels are exceeded *"...food business operators shall review the mitigation measures applied and adjust processes and controls with the aim to achieve levels of acrylamide as low as reasonable achievable below the benchmark levels. Food business operators shall hereby take into account the safety of foodstuffs, specific production and geographic conditions or product characteristics"* (Article 2 (4) of the Regulation (EU) 2017/2158). It also encourages the use of colourimetric scales which provide a fast guidance between the optimal food colour and low levels of acrylamide. Colour guides should be developed for each product type and it is important that they are well displayed in the premises of food production. Furthermore, in Annex III it is stated that "*Analysis of acrylamide can be replaced by measurement of product attributes (e.g. colour) or process parameters provided that a statistical correlation can be demonstrated between the product attributes or process parameters and the acrylamide level*." (Annex III of the Regulation (EU) 2017/2158). This regulation will be applied from 11 April 2018.

From a chemical point of view, acrylamide is an amide of the acrylic acid. It is a difunctional monomer containing a electrophilic double bond and an amine group (see Figure 1). The substance exhibits both weakly acidic and basic properties. The carboxamide group retains electrons and this activates the double bond, which reacts (reversibly) with nucleophilic reagents (such as ammonia, amines, bisulfites, etc.) by 1,4-addition mechanism. Akalinity conditions

allow the addition of mercaptans, sulphides, ketones, nitroalkanes and alcohols (Habermann, 1991).

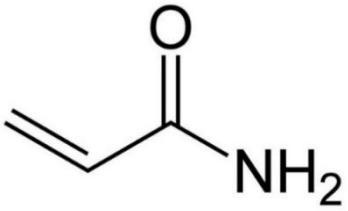

Figure1. Acrylamide chemical structure.

1.2. Acrylamide formation in foods

Acrylamide is formed by several pathways related to the Maillard reaction (Mottram et al., 2002; Standler et al., 2002; Biedermann et al., 2002) in foods containing high concentrations of reducing sugars and free amino acids, especially during cooking processes requiring temperature greater than 120 degrees such as frying, roasting and baking.

Several factors influence acrylamide formation in foods:

- *Type and concentration of reagents*

Asparagine is the most involved amino acid in acrylamide formation during Maillard reaction in starchy foods. In potatoes, for example, asparagine may account for 40% of total amino acid content, while in cereal flour the percentage is 15% (Martin & Ames, 2001; Mottram et al., 2002). Despite the high content of the amino acid, no direct correlation was found between asparagine concentration and acrylamide content in potatoes (Amrein et al., 2003). However, higher amounts of reducing sugars result in a significant acrylamide increase in potato-based foods (Becalski et al., 2004). In the case of cereal products, instead, there is a direct correlation between the formation of the toxic substance and the initial asparagine concentration.

Acrylamide concentration is also strongly dependent on the type of sugar involved in the reaction. Generally, monosaccharides have a greater influence on the acrylamide formation (Zyzak et al,. 2003). Reducing sugars (glucose, fructose, galactose, etc.) can derived from more complex carbohydrates such as disaccharides (sucrose, lactose and maltose) which are easily hydrolyzable at temperatures higher than 100 °C and therefore divided in single monosaccharides after a thermal treatment (Lingnert et al., 2002, Becalaski et al., 2004).

Therefore, reagents that lead to the acrylamide formation are the concentration of reducing sugars (in case of potatoes) and asparagine (in cereal-based foods) (Biedermann et al., 2002; Surdik et al., 2004).

- *Physical chemical characteristics of food matrices*

The pH of the food affects the acrylamide formation because it influences the reactivity of the amine group of the amino acid involved but also of the carbonyl group of reducing sugars. By increasing the pH, the amine group is deprotonated and thus the interaction between amino acid and sugar is favored. By decreasing the pH, the amine group is protonated and this penalizes the formation of the Schiff base (reaction intermediate in the acrylamide formation). The use of acidifying substances allows the reduction of the acrylamide content in foods (Jung et al., 2003; Pedreschi et al., 2004).

Water content of foods also affects the mechanisms of acrylamide formation (Robert et al., 2005; Elmore et al.,2005). Low moisture content (less than 20% water relative to the total weight in potatoes and 10% in bakery products for example) generally tend to increase the final concentration of the toxic substance (Biedermann et al., 2002, Taeymans et al., 2004, Elmore et al., 2005).

- *Process parameters used in food cooking*

The two main process parameters that directly influence the acrylamide development are time and temperature of the heat treatment (Taubert et al., 2004). Parameters such as heat transfer mechanism, heating and cooling rate, shape and thickness of food, cause gradient concentration of acrylamide because food areas subject to higher heating have a higher concentration of this substance (Lingnert et al., 2002).

- *Use of inhibitors in the food formulation*

Several studies have shown that the use of certain compounds reduces acrylamide levels in foods and model systems. For example, it has been shown that addition of amino acids other than asparagine (such as cysteine and methionine) results in acrylamide reduction of 50 % in crackers (Levine and Smith, 2005). Using of baking agents and organic acids have led to a reduction in the acrylamide content in food product models such as semi-finished biscuits (Graf et al., 2006), cracker (Levine and Smith, 2005) and corn chips (Jung et al., 2003). Even employing of natural antioxidants extracted for example from bamboo and green tea leaves could reduce acrylamide levels in foods (Zhang & Zhang, 2008). Finally, it has been shown that in a food model system (fried snack products), water-soluble vitamins reduce the acrylamide formation, while fat-soluble vitamins have only a weak inhibitory effect (Zeng et al., 2009).

Another approach to mitigate acrylamide in foods is the use of asparaginase. This enzymatic method is based on the hydrolysis of asparagine in aspartic acid and ammonia. Satisfactory results have been obtained using asparaginase to reduce acrylamide levels in foods such as potato

products, cereal products and coffee. Studies conducted on these foods are well presented in the review by Xu et al., 2016 (Xu et al., 2016). Despite two types of asparaginase are commercially available (Acrylaway® and PreventASe™), their use in the food industy requires further study. Many factors such as cooking time and temperature, enzyme-substrate ratio and pH must be considered. Furthermore, it is necessary to evaluate whether the treatment with asparaginase can have negative effects on the sensory characteristics of the cooked products.

1.3. Risk assessment for subjects exposed to acrylamide

In 1994 acrylamide was classified as probably carcinogenic to humans by the International Agency for Research on Cancer (IARC, 1994).

A report was published in 2006, where the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and the Joint Expert Committee of Food Additives (JECFA) confirmed that the presence of acrylamide in foods is to be considered a cause for concern (FAO/WHO, 2006). In June 2015, based on animal studies and after a careful risk assessment, the European Food Safety Authority published a scientific opinion on acrylamide in food (EFSA, 2015). EFSA's scientists have confirmed that acrylamide is found in many everyday foods (such as fried potato products, coffee, crackers, biscuits and different types of bread) and it can increase the risk of developing cancer and other effects for consumers in all age groups. The alarm concerns especially children representing the most exposed population group on body weight basis.

It is impossible to establish a tolerable daily intake (TDI) of acrylamide. In fact, this substance is considered genotoxic and carcinogenic and hence at any level of exposure can increase the likelihood of gene mutations and tumors. For this reason, EFSA's experts have defined a confidence interval within which acrylamide is likely to have adverse effects on human health. Based on the lower limit of this range (Benchmark Dose Lower Confidence Limit, $BMDL_{10}$), EFSA's experts have defined the following values:

- $BMDL_{10}$ of 0.17 mg/kg bw/day (milligram per kilogram of body weight per day) for tumours;
- $BMDL_{10}$ of 0.43 mg/kg bw/day (milligram per kilogram of body weight per day) for neurological changes.

Acrylamide levels in foods are constantly monitored by EU Member States and EFSA's scientific advice is important to assess any new measures to reduce population exposure to this substance. Finally, it's important to remember that foods are not the only source of acrylamide. It is also present in tobacco smoke. For smokers and non-smokers (through passive smoke) tobacco smoke is a source of acrylamide exposure greater than food. Acrylamide also has a wide variety of

industrial non-food uses, and therefore, some people may be exposed in the workplace by skin absorption or by inhalation.

In this work the possible correlation between acrylamide content and colour of the product has been evaluated by analysing samples obtained from different cultivar and submitted to different cooking procedures.
Besides, a survey on 30 different chips types purchased form the market was carried out with the aim of evaluating acrylamide content and a possible influence of the harvest time.

2. MATERIALS AND METHODS

2.1. Chemical and reagents

Acrylamide standard (99%), d_3-acrylamide (500 mg/L in acetonitrile) and formic acid (≥98%) were purchased from Sigma-Aldrich (Steinheim, Germany). Methanol and n-Hexan (HPLC grade) were obtained from Carlo Erba Reagents (Milan, Italy). Potassium hexacyanoferrate (Carrez I) and zinc sulphate (Carrez II) were supplied by Merck (Darmstadt, Germany). Deionized water was obtained with a Milli-Q™ system (Millipore, Bedford, MA, USA).

2.2. Samples

2.2.1. Baked potatoes

In this study three different potato varieties (var. Primura, var. Spunta and var. Arizona) found on the market were employed.
Initially, potatoes were peeled, ground and placed with a thickness of about 1cm on the baking tray. Cooking was carried out with "dry air circulation" function and replicated with "moisture 25%" function, using different time (from 30 to 55 minutes) and temperature (from 160 to 200 °C) conditions. After, portions of cooked product (5 x 5 cm) characterized by a different colour were sampled and analyzed to measure acrylamide levels.

2.2.2. Potato chips

32 samples of commercial potato *chips* of the same producer, but different for typology (classic, stick and “rustiche”) were analyzed to measure acrylamide levels. 19 of these samples were obtained using potatoes harvested in Germany at the end of Summer 2016 (“old potatoes”), while the remaining 13 samples were produced from potatoes harvested in Italy at the end of Spring 2017 (“new potatoes”).

2.3. Sample preparation

2.3.1. Preparation of baked potatoes

Acrylamide extraction procedure from baked potatoes without seasoning and oil was developed from a method present in the literature (Calbiani et al., 2004). Briefly, cooked potatoes were treated with liquid nitrogen and ground in a blender. 1 g of sample was weighed in a 50 mL plastic tube and then internal standard solution (100 µL d_3-acrylamide 10 ppm) followed by 0.1% (v / v) formic acid for a final volume of 10 mL were added. After stirring using vortex mixer for 1 minute, extraction was carried out using a magnetic stirrer at room temperature for 10 minutes. Sample was centrifuged (4800 rpm at 4 ° C for 15 min). The obtained supernatant was transferred into a cleaned conical tube and filtered through a 0.45 µm disposable nylon filters prior to instrumental analysis.

2.3.2. Preparation of potato chips

Acrylamide extraction from different types of potato chips was carry out following the method described by Mesias and Morales (Mesias & Morales, 2015) and slightly modified. Potatoes were treated with liquid nitrogen and ground in a blender. 6 g of sample were weighed in a buchner filter and defatted with 250 mL of hexane by a vacuum pump. After eliminating solvent residues, the sample was again weighed and divided into two 0.500 g aliquots in two 50 mL plastic tube. 9.4 mL of formic acid 0.1% (v / v) followed by the internal standard solution (100 µL d_3-acrylamide 10 ppm) were added. After stirring using vortex mixer for 1 minute, extraction was carried out using a magnetic stirrer at room temperature for 10 minutes. Subsequently, sample was treated with 250 µL of Carrez I (150 g / L potassium hexacyanoferrate in water) and 250 µL of Carrez II (300 g / L zinc sulphate in water), stirred using vortex mixer for 1 minute and centrifuged (4800 rpm at 4 ° C for 15 min). The obtained supernatant was transferred into a cleaned conical tube and filtered through a 0.45 µm disposable nylon filters prior to instrumental analysis.

2.4. Instrumental analytical method

The instrumental analysis was performed on an Agilent 1200 liquid chromatograph coupled to a mass spectrometer, equipped with an electrospray (ESI) interface and a linear ion trap analyzer (LC/MSD Trap XCT Ultra, Agilent Technologies). LC separation was carried out on a Luna C18 column (250 mm × 2 mm, 5µnm particle size; Phenomenex Inc., CA, USA) thermostated at 30 °C using an injection volume of 10 µL and a gradient solvent elution system composed by:

- (A) 99.5: 0.5 formic acid 0.1%: methanol (v/v)
- (B) 100 % methanol

Table 1 shows the gradient elution used:

Table 1. Gradient elution

Time (min)	% B	Flow (mL)
0.00	0.0	0.200
10.00	0.0	0.200
12.00	80.0	0.250
24.00	80.0	0.250
26.00	0.0	0.200
40.00	0.0	0.200

For data acquisition electrospray ionization was used in positive mode (ESI +). The nebulizer gas (nitrogen, 99,9% purity) and the dry gas (nitrogen, 99,9% purity) were delivered at flow rates of 60.0 psi and 10.0 L/min, respectively. Other conditions of the interface were: ESI voltage 4.5 kV, skimmer voltage 40.0 V, dry temperature 365 °C. Masses were recorded in SIM (Selected Ion Monitoring) mode, selecting signals of molecular ions $[M+H]^+$ at m/z 72 and m/z 75 for acrylamide and d_3-acrylamide, respectively. Confirmation of acrylamide identity was made by a comparison of migration time and mass spectrum obtained from sample and standard solution.
Agilent ChemStation B.01.03 and LC–MSD Trap 6.0 Build 458.0 softwares were used to control the instrument and for data processing.

2.5. Method validation

In order to validate both analytical methods employed for acrylamide quantification, limit of detection (LOD), limit of quantification (LOQ), linearity, accuracy (precision and trueness) were calculated according to Eurachem guidelines (EURACHEM Guide, 1998). For this reason, potatoes boiled were used as blank matrix after excluding the presence of acrylamide.
LOD and LOQ values were calculated from the matrix calibration curve as 3 *s*/slope and 10 *s*/slope, respectively, where *s* is the standard deviation of the blank signal obtained from ten independent blank measurements.
Linear range was investigated starting

- from LOQ value to 12500 ng/g (6 concentration levels) for baked potatoes;
- from LOQ value to 3200 ng/g (5 concentration levels) for potato crisps.

After variance homogeneity assessment ($p>0.05$) and testing significance of the intercept ($p<0.05$ at 95% confidence level), linearity was also mathematically verified by applying the Mandel fitting test ($p>0.01$ at the 99% confidence level). Quantitative analysis was performed using the calibration curve built in matrix by internal standard method. The same amount of

d_3-acrylamide, as internal standard, was added in each solution to reach the final concentration of 100 ng/mL. The analytical signal considered was the value of peak area/peak area of internal standard (A/AIS).

Accuracy was assessed in terms of precision and trueness. Precision was assessed as RSD% in terms of intra-day repeatability and intermediate precision (inter-day repeatability) of migration times and peak areas. For this purpose, the within-day repeatability was evaluated by performing three independent extractions of blank matrix fortified with acrylamide standard solutions at 1000 ng/g concentration level and three injections for each sample in the same day. The inter-day repeatability was calculated on five days by performing five independent extractions of the blank matrix fortified with acrylamide standard solution of 1000 ng/g concentration level and three injections per day for each sample.

Trueness was evaluated in terms of recovery by analyzing in triplicate blank matrix samples fortified before extraction at 5000 ng/g and 3200 ng/g concentration levels (in the case of research work about baked potatoes and potato chips, respectively). Percentage of recovery was then calculated with the formula:

Recovery (%) = [(C1-C2)/C3] x 100

where C1 is concentration determined in fortified sample, C2 is concentration determined in unfortified sample (in this case, C2=0 since blank matrix did not contain acrylamide) and C3 is concentration of fortification.

3. RESULTS AND DISCUSSION

3.1. Analysis conditions

For chromatographic separation, the method proposed by Calbiani et al., 2004 was employed, slightly revisited. In particular, after elution of acrylamide (6.1 min) under isocratic conditions (100% solvent A, 0.200 mL / min for 10 min), it was decided to increase solvent B as follows: it was delivered from 0% to 80% in 2 min and maintained in this percentage for 12 min (0.250 mL / min) before column re-equilibration for 16 min (0.200 mL / min). In this way it was possible to wash the column without making a “blank analysis” between the analysis of two consecutive samples.

Furthermore, by direct infusion of acrylamide and d_3-acrylamide at a 10 ppm concentration level, some parameters of the ESI source were optimized to improve analyte ionization. In particular *Dry temperatures*, *Spray Voltage* and *Gas Nebulizers* were parameters that influenced ionization (due to preliminary experiments). Dry temperature was changed between 320 and 365 °C, and

after appropriate tests the highest value (365 °C) was set, which allowed an increase in the acrylamide signal by better vaporization of the solvent. Nebulizer gas was changed between 30-60 psi and the optimal condition, from a sensitivity point of view, was found to be the highest value (60 psi). Spray voltage was changed between 3.5-4.5 kV and was set to the highest value (4.5 kV) since it provided the best performance in terms of signal intensity.

3.1. Sample preparation

In the first part of the research work, the method used to extract acrylamide from the samples was defined. The remarkable difference between baked potatoes (without seasoning and oil) and potato chips has requested the use of two extractive protocols.

3.1.1. Preparation of baked potatoes

In literature there are many scientific articles about the extraction of acrylamide from food matrices. Initially, the treatment of the sample was performed using the method described by U.S. Food and Drug Administration (U. S. Food and Drug Administration, 2003). The main steps of this method included extraction of acrylamide (added as internal standard, d3-acrylamide) from the matrix using water and a subsequent cleanup with two SPE cartridges (Oasis® HLB and Bond Elut-Accucat®). Although these phases are usually employed in protocols to extract acrylamide from food (Tareke et al., 2002; Becalski et al., 2004; Govaert et al., 2006), the FDA method did not give good results in terms of recovery and repeatability of the analysis. The SPE step was then eliminated and thanks to the simplicity of the food matrix (baked potatoes without condiments), it was decided to extract the acrylamide on the basis of its solubility in water. For this reason the samples were treated in agreement with Calbiani et al.,(2004), obtaining high recovery.

3.1.2. Preparation of potato chips

The presence of oil in these samples has initially complicated the acrylamide extraction process, making impossible the use of the quick and easy method followed for baked potatoes. The method proposed by Mesias and Morales (Mesias & Morales, 2005) was then employed and slightly modified as described in the Materials and Methods section (*2.3.2 Preparation of potato chips*), obtaining a satisfactory recovery.

3.2. Method validation

Method validation for quantitative purposes was conducted according to Eurachem guidelines (EURACHEM Guide, 1998), as reported in Material and Methods section. Obtained data are shown in Tables 2 and 3.

Table 2. Method validation results obtained in matrix for baked potatoes

Standard	LOD, LOQ (ng/g)	Linearity range (ng/g)	Calibration curve	R^2	Trueness %	Intra-day rep (area, RT)	Inter-day rep (area, RT)
Acrylamide	225, 750	750-12500	y=0.001x	0.981	99 ± 1	3.1%, 0.8%	4.1%, 1.4%

Table 3. Method validation results obtained in matrix for potato chips

Standard	LOD, LOQ (ng/g)	Linearity range (ng/g)	Calibration curve	R^2	Trueness %	Intra-day rep (area, RT)	Inter-day rep (area, RT)
Acrylamide	180, 600	600-3200	y=0.001x-0.068	0.990	92 ± 2	5.3%, 1.1%	6.0%, 1.3%

3.3. Samples analysis

3.3.1. Acrylamide levels in baked potatoes

The purpose of this part of research, in collaboration with Art Joins Nutrition (Dr. Chiara Manzi), was carried out on mashed baked potatoes was to investigate if similar colours obtained under different cooking conditions, indicated similar amounts of acrylamide.

Cooking was performed in domestic ovens using different time / temperature combinations. A wide range of colours was achieved (from lightest to darkest, representing an undercooked and a burnt product, respectively), simulating all the possible colourations of the product that consumers can obtain. In particular, 11 different colours have been identified, both with the dry air circulation function (see Figure 2) and with the moisture 25% (see Figure 3). The cooked product portions (squares of size 5x5) were sampled from different areas of the same baking tray and subsequently analyzed to quantify the acrylamide levels. Table 4 shows the quantities of acrylamide found in the analyzed samples.

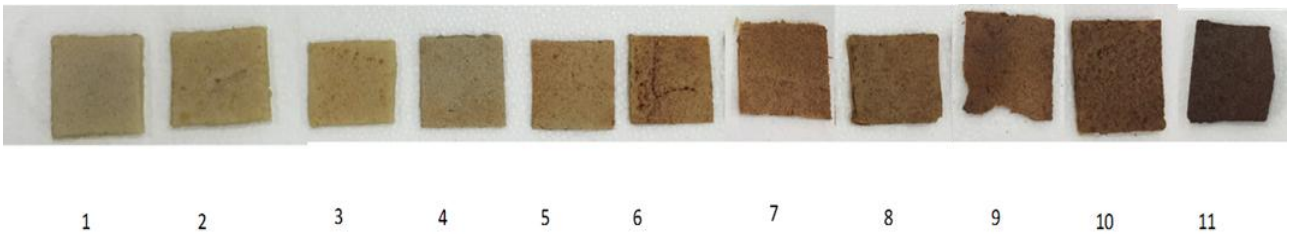

Figure 2. Colors identified using dry air circulation function

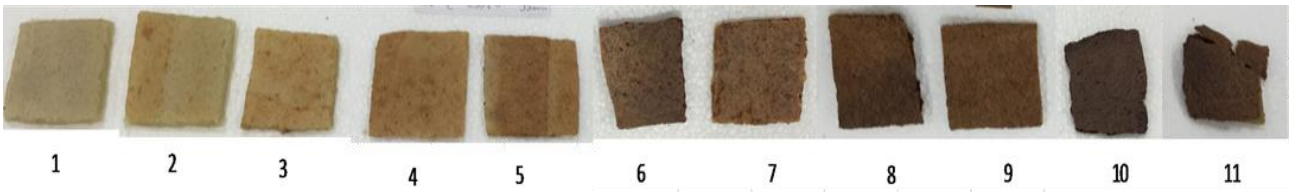

Figure 3. Colours identified using moisture 25%

In Table 4 samples are enumerated in ascending order (from lightest to darkest) and the existence of a correlation between colour intensity and acrylamide content could be evidenced. In contrast to what reported in a scientific work on baked bread with and without steam (Ahrné et al., 2007), in our case, steam cooking (moisture 25%) did not reduce the acrylamide level in the product. It is true that steamed cooking should increase the moisture content of the product by reducing the formation of the toxic substance (Biedermann et al., 2002, Taeymans et al., 2004, Elmore et al., 2005), but probably the type of food matrix and the different heat transfer of the moisture and the dry air on the surface of the product play a crucial role. It can be assumed that, in the presence of moisture, a greater acrylamide content is formed since the steam condenses on the surface of the food that initially has a lower temperature. Since water is the main solvent in which water-soluble macro- and micro-elements occur, steam (pure water) goes into the product by osmosis enhancing the availability of substrates. In this way, in presence of high temperatures, the Maillard reaction and the development of acrylamide are favoured.

Moreover, the higher levels of acrylamide found in steam-cooked potatoes (together with a greater browning) could depend on the higher temperature reached on the surface of the food due to a more effective heat transfer of steam respect to dry air.

Table 4. Acrylamide levels in mashed baked potatoes employing two oven functions

Sample number	Acrylamide content (ng/g)	
	Dry air circulation	Moisture 25%
1	n. d	n. d
2	n. d	n. d
3	n. d	n. d
4	n. d	389 ± 1 (*)
5	269 ± 19 (*)	671 ± 48 (*)
6	269 ± 70 (*)	860 ± 10
7	990 ± 30	1069 ± 530
8	972 ± 38	1346 ± 17
9	1077 ± 710	7562 ± 21
10	2993 ± 49	9550 ± 550
11	9820 ± 320	10998 ± 140

(*) < LOQ

Colour analyses performed in the Department of Food and Drug (Dr. Massimiliano Rinaldi) on the same samples, on the basis of the colour channels Red, Green and Blue, showed that acrylamide increase was accompanied by a darker colour (see Tables 5 and 6).

Table 5. Colour analysis performed on samples cooked with dry air circulation

Sample number	DRY AIR CIRCULATION		
	RED	GREEN	BLUE
1	129,32 ± 5,18	116,86 ± 5,02	88,5 ± 5,03
2	136,71± 7,08	117,91 ± 7,57	77,89 ± 7,76
3	144,53± 5,13	121,78 ± 5,72	76,78 ± 6,12
4	136,04± 7,64	121,77 ± 8	88,57 ± 8,29
5	139,27± 8,94	111,44 ± 9,67	71,12 ± 9,61
6	126± 15,6	92,23 ± 16,17	56,82 ± 13,54
7	120,87± 12,43	84,11 ± 12,41	53,86 ± 11,34
8	109,46± 9,24	81,2 ± 9,43	52,69 ± 8,96
9	95,15± 13,55	64,95 ± 12,22	43,51 ± 9,4
10	88,92± 10,27	61,77 ± 10,25	43,42 ± 9,68
11	62,28± 9,35	46,32 ± 9,27	38,96 ± 8,81

Table 6. Colour analysis performed on samples cooked with moisture 25%

Sample number	MOISTURE 25%		
	RED	GREEN	BLUE
1	149,71 ± 5,43	133,1 ± 5,26	99,62 ± 5,29
2	150,54 ± 5,42	128,71 ± 7,31	85,3 ± 9,65
3	156,13 ± 7,1	125,11 ± 8,82	76,63 ± 8,93
4	139,32 ± 8,02	105,14 ± 8,32	64,4 ± 7,51
5	128,93 ± 9,38	93,45 ± 10,24	58,18 ± 8,91
6	105,16 ± 11,81	78,64 ± 12,13	54,34 ± 11,35
7	92,33 ± 14,43	70,27 ± 13,7	51 ± 11,53
8	94,04 ± 9,04	66,26 ± 8,75	43,42 ± 8,08
9	76,39 ± 10,86	53,83 ± 9,45	37,53 ± 8,37
10	67,13 ± 8,61	52,06 ± 8,44	42,95 ± 8,09
11	58,61 ± 7,95	43,28 ± 6,94	34,54 ± 6,11

In the tables above it can be seen that as the RGB values decrease, the colour of the sample becomes more dark and consequently the content of acrylamide increases.

Data processing obtained from the acrylamide analysis and the colour values has allowed to calculate a degree of correlation between the intensity of the colour channels (R, G, and B) and the acrylamide content, with a coefficient R^2 equal to 0.844, using the following equation:

$$acrylamide\left(^{\mu g}/_{g}\right) = 15.97 - 0.211 * R + 0.028 * G + 0.134 * B$$

Thus, the observed colour gradation can be considered as a marker that can give information on the acrylamide level in the food product (Pedreschi et al., 2007).

3.3.2. Acrylamide levels in potato chips

In this second part of the research (in progress) the acrylamide level in commercially available chips was determined. The analysed samples were different not only for type (classic, stick and "rustiche"), but also for harvest time (Summer and Spring potatoes) and for origin (Germany and Italy) of the raw material. Data are shown in Tables 7 and 8.

Table 7. "Old potatoes" (German)

Sample		Acrylamide amount (ng/g)	Fat removed (%)
Code	**Type**		
C1	classic	217 ± 10 (*)	32
C2	classic	540 ± 13 (*)	33
C3	classic	609 ± 13	15
C4	classic	744 ± 31	33
C5	classic	821 ± 31	32
C6	classic	472 ± 58 (*)	31
C7	classic	929 ± 26	12
C8	classic	398 ± 32 (*)	31
C9	classic	900 ± 14	32
C10	classic	1139 ± 117	27
C11	classic	n. d	29
R1	rustiche	633 ± 71	24
R2	rustiche	553 ± 13 (*)	15
R3	rustiche	541 ± 67 (*)	29
R4	rustiche	336 ± 21 (*)	30
R5	rustiche	457 ± 47 (*)	29
R6	rustiche	n.d	18
S1	stick	536 ± 19 (*)	26
S2	stick	657 ± 65	19

(*) < LOQ

Table 8. "New potatoes" (Italian)

Sample		Acrylamide amount (ng/g)	Fat removed (%)
Code	**Type**		
C1	classic	449 ± 51 (*)	21
C2	classic	614 ± 7	30
C3	classic	841 ± 51	26
C4	classic	252 ± 23 (*)	39
C5	classic	244 ± 6 (*)	34
C6	classic	699 ± 15	34
C7	classic	575 ± 38 (*)	30
C8	classic	373 ±51 (*)	24
C9	classic	n. d	27
C10	classic	264 ± 25 (*)	33
C11	classic	n. d	28
C12	classic	181 ± 6 (*)	32
R1	rustiche	n. d	28

(*) < LOQ

As can be seen from the tables above, chips type does not appear to affect the amount of oil absorbed during frying. Classic chips seem to show higher levels of acrylamide than "rustiche" and stick.

Regardless of type, potatoes harvested at the end of Summer and stored until processing (see Table 7) show average amounts of acrylamide higher than potatoes harvested at the end of Spring and immediately processed (see Table 8) (552 ng/g ± 293 e 345 ng/g ± 276, respectively). This is in accordance with literature (Ohara-Takada et al., 2005); in fact, potatoes stored for longer time are characterized by a higher acrylamide content in the final product.

4. CONCLUSIONS

From the study on baked potatoes it has been revealed that the presence of moisture (25%) involves an increase of acrylamide levels in the product compared with traditional cooking. This has been attributed to the greater heat transfer of steam respect to the dry air, which usually results in a higher temperature on the food product surface.

The existence of a correlation between the acrylamide content and the color of the product has been confirmed, regardless of the time / temperature combination of cooking.

Regarding potato chips analysis, the collected data confirm scientific literature findings as longer storage times involve a greater amount of acrylamide in the processed product, probably due to an increase of the simple sugars amount in the raw material.

It would be interesting to develop this research by selecting cultivars with low amounts of reducing sugars or asparagine, and by optimizing harvest time, storage conditions, and technological processes to minimize acrylamide formation.

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CHAPTER 3: STUDY ON SAMPLE PREPARATION BY CLOUD POINT EXTRACTION (CPE) FOR NANOPARTICLES INVESTIGATION

1. INTRODUCTION

1.1. Nanotechnology

"Nanoscience is the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales, where properties differ significantly from those at a larger scale. Nanotechnologies are the design, characterisation, production and application of structures, devices and systems by controlling shape and size at nanometre scale." (The Royal Society & The Royal Academy of Engineering, 2004).

The first concept about Nanoscience and Nanotechnology was introduced in 1959 by the physicist Richard Feynman, during his talk "There's Plenty of Room at the Bottom" at an American Physical Society meeting at the California Institute of Technology. Feynman explored the possibility of manipulating material at the scale of individual atoms and molecules, imagining the whole of the Encyclopaedia Britannica written on the head of a pin and foreseeing the increasing ability to examine and control matter at the nanoscale (The Royal Society & The Royal Academy of Engineering, 2004). Several years later, in 1974, the Japanese researcher Norio Taniguchi coined the term "nanotechnology" thanks to his explorations of ultraprecision machining.

Nanotechnology is often difficult to define, because the complex field of the matter´s manipulation at the nanoscale can be considered from the physical, chemical, biological and engineering point of view. For this reason, at the international level, there are many definitions of "nanotechnology". For example, according to the The Royal Society "Nanotechnologies are the design, characterisation, production and application of structures, devices and systems by controlling shape and size at nanometre scale" (The Royal Society & The Royal Academy of Engineering, 2004). The International Organization for Standardization (ISO) defines nanotechnology as follows: "application of scientific knowledge to manipulate and control matter predominantly in the nanoscale to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material" (ISO/TS 80004-1:2015). The European Commission (EC) provides the following definition: "Nanotechnologies are technologies dealing with dimensions measured in billionths of a meter, or nanometres (nm)" (www.ec.europa.eu). According to the European Food Safety Authority (EFSA) "Nanotechnology is a field of applied sciences and technologies involving the control of matter on the atomic and molecular scale, normally below 100 nanometers" (www.efsa.europa.eu). On the web site of the U.S Food & Drug Administration (FDA) there is not a definition for nanotechnology. "However, when scientists talk about

nanotechnology they are usually referring to the manipulation of material of extremely small size, usually at dimensions between 1 and 100 nanometers." (www.fda.gov).

These are just some definitions of "nanotechnology", but most of them highlight two fundamental principles: size and unique properties (Shatkin, 2012). Features of structures and systems at nanoscale are different from the macro-scale counterpart due to the interactions of individual atoms and molecules and all this offers new applications (Neethiarajan & Jayas, 2011). For example, when the size of the matter is at the nanometer scale, electrical, optical and mechanical properties are different from bulk material or larger sized material, due to the increase in the surface/ volume ratio.

Due to its interdisciplinary nature, nanotechnology is applied in different areas of society such food industry, agriculture, cosmetics, electronics, medical and health. Development and production of nanosized particles, fibers, coatings, etc., in general termed nanomaterials, needs to be regulated to protect the environment and human health.

Furthermore, to investigate the chances and risks of "nanotechnology" it is essential to develop methods for the detection, measurement, quantification and characterisation of nanomaterials.

1.2. Nanomaterials

1.2.1. Definition of nanomaterials

Like Nanotechnology, there are numerous definitions for nanomaterials because they are applied in different area of life.

In general, the term "*nanomaterial"* refers to materials or objects having dimensions in the range of nanoscale. The International Organization for Standardization (ISO) defines "*nanoscale*" as the length range approximately from 1 nm to 100 nm (ISO/TS 80004-1:2015) including in the generic term "nanomaterial" both "nano-objects" and "nanostructured materials" (see Figure 1).

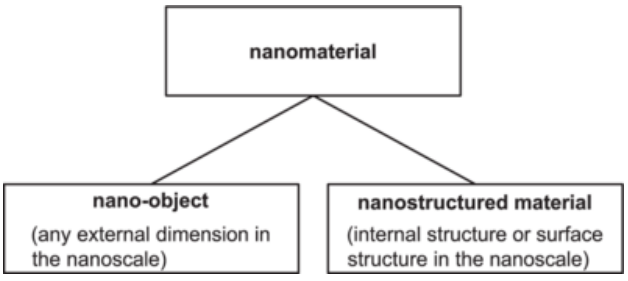

Figure 1. Nanomaterial framework (ISO/TS 80004-1:2015).

"Nano-objects" have one or more external dimensions in the nanoscale (see Figure 2) and show different chemical reactivity, optical, physical, magnetic, electrical, mechanical and toxicological proprieties from their macroscale counterpart as a result of their small size. Furthermore, due to surface interactions, "nano-objects" can be assembled, resulting in agglomerates and/or aggregates.

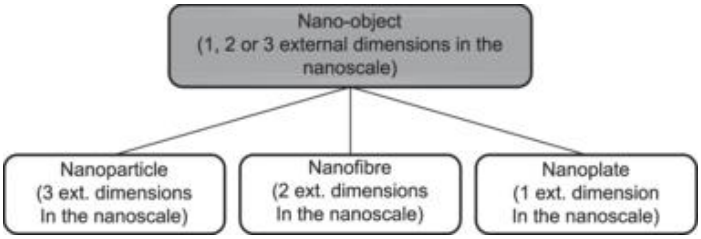

Figure 2. Fragment of hierarchy of terms related to nano-object (ISO/TS 80004-2:2015).

The ISO definition is the only one that could be considered internationally, but the exclusive focus on size is often insufficient to define a "nanomaterial".

The lack of a comprehensive and universally recognized definition often involves difficulty and confusion in determining whether a material is or is not a nanomaterial. For example, the size limits and other properties that delineate a "nanomaterial" are somewhat arbitrary (Table 1).

Table 1. Comparative assessment of elements considered in current and advisory definitions of "nanomaterials" (Boverhof et al., 2015).

Organization and/or regulation	Size	Solubility	Aggregates and agglomerates	Distribution threshold	Intentionally manufactured/ engineered	Novel properties
European Commission Cosmetics Directive	1 - 100 nm	Yes	Yes	No specific mention	Yes	No
Australian Government Department of Health and Ageing	1 - 100 nm	No	Yes	10% by number	Yes	Yes
Health Canada	1 - 100 nm and larger[a]	No	Yes	No specific mention	Yes	Yes
United States Food and Drug Administration	1 - 100 nm and larger[b]	No	No specific mention	No specific mention	Yes	Yes
United States Environmental Protection Agency	1 - 100 nm	No	Yes	10% by weight	Yes	Yes
European Commission Recommendation for a Definition	1 - 100 nm	No	Yes	50% by number	No	No
European Parliament and the Council of the European Union on the Provision of Food Information to Consumers	1 - 100 nm and larger[a]	No	Yes	No specific mention	Yes	Yes
European Commission Biocides Directive	1 - 100 nm	No	Yes	50% by number	No	No
French Ministry of Ecology, Sustainable Development, Transport and Housing	1 - 100 nm	No	Yes	50% by number	Yes	No
Taiwan Council of Labor Affairs	1 - 100 nm and larger[a]	No	No specific mention	No specific mention	Yes	Yes
Swiss Federal Office of Public Health and Federal Office for Environment	1 - 100 nm and larger[c]	No	Yes	50% by number	No	No
Norwegian Environment Agency	1 - 100 nm and larger	No	Yes	50% by number	No	No
Belgian Federal Public Service Health, Food Chain Safety and Environment (Belgium, 2014)	1 - 100 nm	No	Yes	50% by number	Yes	No
Danish Ministry of the Environment (2014)	1 - 100 nm	No	Yes	50% by number	No	No

[a] Health Canada, the European Commission (for food and food contact materials) and the Taiwan Council of Labor Affairs have indicated the inclusion of materials larger than the nanoscale in all dimensions if they exhibit one or more nanoscale properties/phenomena.
[b] The US FDA includes materials up to one micron if the material exhibits properties or phenomena that are attributable to its dimensions.
[c] The Swiss definition includes substances with primary particles, aggregates and agglomerates up to 500 nm, as well as respirable materials of up to 10 microns that may have nanoscale side branches.

At the lower end of the range, the chemistry of the molecules defines the properties of the materials. At the upper end instead, nanoscale properties such as solubility, light scattering and surface area effects, are predictable and continuous characteristics of the bulk materials (Donaldson & Poland, 2013).

An alignment between definitions is very difficult, because the classification of nanomaterials according to quantitative criteria requires a validated measurement method. There are different

reliable methods to measure isolated nanomaterials. But up to now there is no standardized method to identify or measure nanomaterials in complex media.

Regardless of the definition used, it is important to evaluate the risk that nanomaterials can represent for the environment and human health. In addition, the lifetime of the nanomaterials should be considered, because aggregation, agglomeration and degradation phenomena or changes in physicochemical characteristics may occur. Therefore, a careful evaluation should also include the potential exposure to the material (for example during production, use and disposal) and not only its potential hazard.

1.2.2. Nanomaterial definition by the European Commission and the first inclusion of nanomaterials in a regulation

In October 2011 the European Commission published a recommendation on a definition of nanomaterial based on the reference report of the Joint Research Center (Lövestam et al., 2010) and the scientific opinion of the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR, 2010).

According to the European Commission (Commission Recommendation No. 2011/696/EU, point 2):

"2. 'Nanomaterial' means a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm.

In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 % may be replaced by a threshold between 1 and 50 %."

It is important to highlight that this is only a recommendation and therefore it is not legally binding. It should be used as a reference for legislative and policy purposes in the European Union and it can be adapted to different regulatory sector.

Currently, there is no legislation entirely dedicated to regulation of nanomaterials. For this reason, nanomaterials are defined (in more or less explicit form) in the regulatory framework of the field in which they are applied (Bleeker et al., 2013).

Before the adoption of the recommendation by the European Commission, a nanomaterial definition had been included in the regulation on cosmetic products (Regulation (EC) No. 1223/2009). This legislation describes nanomaterials as an "*insoluble or biopersistent and intentionally manufactured material with one or more external dimensions, or an internal*

structure, on the scale from 1 to 100 nm." (Regulation (EC) No. 1223/2009; article 2, paragraph 1 k).

1.2.3. Regulatory aspects of nanomaterials: focus on food

The Novel Food Regulation revised in 2015, defines *"engineered nanomaterial"* as *any intentionally produced material that has one or more dimensions of the order of 100 nm or less or that is composed of discrete functional parts, either internally or at the surface, many of which have one or more dimensions of the order of 100 nm or less, including structures, agglomerates or aggregates, which may have a size above the order of 100 nm but retain properties that are characteristic of the nanoscale. Properties that are characteristic of the nanoscale include: (i) those related to the large specific surface area of the materials considered; and/or (ii) specific physico-chemical properties that are different from those of the non-nanoform of the same material* (Regulation (EU) 2015/2283; article 3 (f)). For this regulation, not only foods but also vitamins, minerals and other substances containing engineered nanomaterials are considered novel foods.

The same definition of "engineered nanomaterials" is currently part of the EU Regulation 1169/2011 on the Provision of Food Information to Consumers, which is amended by EU Regulation 2015/2283. In fact, in the Novel Food Regulation (Regulation (EU) 2015/2283; point 10) it is stated that *"... The appropriate legislative framework for including such a definition is this Regulation. Accordingly, the definition of engineered nanomaterial, along with the related conferral of delegated powers to the Commission, should be deleted from Regulation (EU) No 1169/2011 and replaced by a reference to the definition set out in this Regulation. Furthermore, this Regulation should provide that the Commission should, by means of delegated acts, adjust and adapt the definition of engineered nanomaterial set out in this Regulation to technical and scientific progress or to definitions agreed at international level."* Nevertheless, the EU Regulation 1169/2011 is important, because it has stipulated that from 13 December 2016 *"All ingredients present in the form of engineered nanomaterials shall be clearly indicated in the list of ingredients. The names of such ingredients shall be followed by the word 'nano' in brackets"* (Article 18 (3) of the Regulation (EU) 1169/2011).

Substances which are intentionally added to food for technological purposes are defined "food additives" and are covered by Regulation (EC) No. 1333/2008. According to this Regulation, all food additives must be authorized and listed in the Community list (Article 1 (a) of the Regulation (EC) No 1333/2008). Furthermore *"When a food additive is already included in a Community list and there is a significant change in its production methods or in the starting*

materials used, or there is a change in particle size, for example through nanotechnology, the food additive prepared by those new methods or materials shall be considered as a different additive and a new entry in the Community lists or a change in the specifications shall be required before it can be placed on the market" (Article 12 of the Regulation (EC) No 1333/2008). Currently, all food additives permitted before 2009 are subject to a reassessment program about their safety by EFSA, in accordance with Regulation (EU) 257/2010 (Commission Regulation (EU) No. 257/2010). It is not explicit in the Regulation (EU) 257/2010, but this re-evaluation has been applied and will also apply to substances having the constituent particle size or a fraction of these in the nanoscale. This new safety evaluation of all food additives is expected to be completed in 2020 (http://www.efsa.europa.eu/en/topics/topic/food-additive-re-evaluations).

All materials and articles intended to come into contact with food are generally defined as Food Contact Materials and are covered by Regulation (EC) 1935/2004. Although not explicitly mentioned, food contact materials containing nanomaterials fall in this regulation.

Regulation (EC) 450/2009 on active and intelligent materials and articles intended to come into contact with food refers to nanomaterials in a rather general way and in fact it states that *"… New technologies that engineer substances in particle size that exhibit chemical and physical properties that significantly differ from those at a larger scale, for example, nanoparticles, should be assessed on a case-by-case basis as regards their risk until more information is known about such new technology. …"* (point 14, Commission Regulation (EC) 450/2009). Instead, Regulation (EU) 10/2011 on plastic materials and articles intended to come into contact with food is more specific because it states that *"Substances in nanoform shall only be used if explicitly authorised and mentioned in the specifications in Annex I."* (Article 9 (2) Commission Regulation (EU) 10/2011).

According to Regulation (EU) 10/2011 (point 27) and Regulation (EC) 450/2009 (point 14), nanoparticle *"should not be covered by the functional barrier concept".* A functional barrier is a layer inside the food contact material that avoids the migration of substances from behind the barrier into the food. *"Behind a functional barrier, non-authorised substances may be used, provided they fulfil certain criteria and their migration remains below a given detection limit"* (Commission Regulation (EC) 450/2009 (point 14); Commission Regulation (EU) 10/2011 (point 27)).

1.3. Nanotechnology and food sector

In the last few years, many food companies are conducting research projects to improve production process and food quality. In this perspective, nanotechnology could be a tool for the food industry where competition and innovation are very important.

Potential applications of nanotechnology can be found at all stages of the food chain.

In agricultural production, improving efficacy of agrochemicals, nanotechnology can offer the possibility to have a better management of pests and crop adherence. For example, pesticides or herbicides can be nano-encapsulated as a more effective delivery and target system (Kah et al., 2012; Sekon, 2014; Axelos & Van de Vorde, 2017). Another possibility is to incorporate these substances and other agrochemicals (such as fertilizers) into nano-emulsions for their controlled release, reducing the amounts that have to be employed (Wang et al., 2007; Liu et al., 2006; Pandey et al., 2010).

Other applications in the agri-food sector are related to the use of nanotechnologies to eliminate possible contaminants (such as pesticides) from water and soil (Bouwmeester et al., 2009; FAO, 2010).

Studies have also been conducted to understand the possible application of nanotechnology in veterinary medicine, for example to replace antibiotics (Fondevila et al., 2009; Pineda ed al., 2012 a, b) and to reduce the toxicity of mycotoxins that often contaminate feed causing diseases in animals (YingHua et al., 2005; Shi et al., 2006).

Except nanomaterials (in terms of biopolymers like carbohydrates, proteins and fats) naturally present in food matrices and nanostructures (such as casein micelles or fat globules) which are formed as a result of food processing operations, nanotechnology can be employed in different ways in food processing. One way is increasing the bioavailability of bioactive compounds, thanks to their encapsulation (Taylor et al., 2005). This strategy offers the possibility to have other applications, such as improving nutrient stability during processing and shelf life of products, masking taste and flavor, retaining volatile substances, and increasing nutritional value of foods (Neethirajan & Jayas, 2011; Chushen et al., 2012).

Another nanotechnology application involves food additives; in fact, some of these substances (such as anti-caking agents or whiteners) contain nanoparticles to increase, for example, the brightness of confectionery products.

In addition, reducing the size of the droplets in an emulsion to nanoscale, it could be possible to improve the nutritional value of existing products, giving the consumer a healthier alternative (such as creamy products, but with a low fat content) (Chaudhry et al., 2008).

Currently, food packaging is the main area for nanotechnology application in the food field; (Chaudhry et al., 2008). thanks to the development of innovative packaging materials, there are several possibilities to increase shelf life of foods and to provide safe products to consumers. For example, an alternative to conventional polymers is represented by nanocomposites, obtained by incorporating nanomaterials as fillers in the polymer matrix. In this way packaging characteristics such as flexibility, barrier properties (against gas, moisture, flavour/compound aroma, UV radiation) and resistance (thermal and mechanical) are improved. Other important features such as density and transparency do not undergo significant changes because nanofillers are usually used in small quantities (in general lower than 5 % by weight) (Cushen et al., 2012; Greiner et al., 2015).

"Intelligent" or "smart" packaging provides information on the quality of the packaged product, by incorporating into packaging material nanostructures (such as sensors, indicators or biochips) with colorimetric properties. Thus, foods could be checked during transport and storage, identifying changes in environmental conditions or microbial and / or chemical contaminants. Therefore, the consumer observing the colour of the sensor can decide if the product can be consumed (Ducan, 2011). These systems could also be used to reduce food waste. In fact, some foods are still good for consumption after the expiration date and employing smart packaging these products can be consumed until they are spoiled or contaminated (Greiner et al., 2015).

Nano-devices can be placed on the food or on the packaging to follow the product across the food chain. Several systems have been developed and represent future applications that can help food industries in tracking, tracing and brand protection. Examples are nanobarcodes (metallic nanoparticles strips that are read by a scanner), nanodisks (functionalized with chromophores that emit a unique light spectrum when illuminated with a laser beam) and Radio Frequency Identification Display (RFID) tags (microprocessors that thanks to an antenna send information to a wireless receiver) (Cushen et al., 2012; Greiner et al., 2015).

In addition, with the aim of producing less waste and thus reducing environmental pollution, there is great interest in edible coating and biodegradable packaging development. Edible coatings made from natural ingredients (such as polysaccharides, proteins and lipids) have been designed for different foods such as meat, cheese, fruits and vegetables. Due to the good barrier properties and the ability to contain substances like antioxidants, these thin films could increase the shelf life of foods, preserving organoleptic characteristics. The disadvantage of biodegradable packaging is that they are usually lacking in stability and barrier properties compared to conventional counterparts. These features are improved thanks to the use of nanomaterials in the natural polymer matrix (such as starch, chitin).

Some packaging materials can extend shelf life and maintain food safety by actively modifying the conditions of the packaged product. In particular, these packaging systems termed "active" can regulate the qualitative and quantitative composition of the head space due to particular barrier properties and/or permeability against gas and moisture; in addition they can absorb oxygen or reduce the growth rate of microorganisms due to the presence of antioxidant or antimicrobial compounds incorporated in the packaging material. In this way, active packaging can be used to improve food quality by preventing microbiological, enzymatic and chemical-physical degradation phenomena and better preserving sensory and nutritional characteristics of products.

Nanotechnology offers the opportunity to revolutionize food industry but many of the applications mentioned above are still in a developmental stage. Nevertheless, in the document "Inventory of nanotechnology applications in the agricultural, feed and food sector" (EFSA Supporting publication 2014:EN-621) (Peters et al., 2014), it has been highlighted that 276 nanomaterials are present on the market. Unfortunately, due to a lack of transparency, it is currently impossible to quantify the real use of nanotechnology in the food field.

However, extensive research is necessary to expand the knowledge on the behaviour of nanomaterials, in terms of possible migration from packaging materials, interaction with food matrix, possible uptake and physiological effects. All this is important for a proper risk assessment, although there are many difficulties from analytical point of view. In fact, nanomaterials characterization can be made on the basis of different chemical-physical parameters such as shape, morphology, surface charge, aggregation/agglomeration, particle size and many others, which can not be determined using only one analytical technique (Picó, 2016). Moreover, sample preparation is often challenging, both for the complexity of the food matrix and the lack of validated procedures.

The here presented study is related to the development of appropriate preliminary sample preparation methods for nanomaterials characterization into food. The object of research were silver nanoparticles (AgNPs), an example of materials that could be find in many consumer products due to their widespread use as antimicrobial (McGillicuddy et al., 2017).

For this property AgNPs are also included within polymer matrices for food packaging (Carbone et al., 2016) with the purpose of increasing shelf life of packaged products, inhibiting the growth of microorganisms. In Europe, however, these nanoparticles are not approved for use in the food sector, since the toxicological data currently available do not allow for a proper risk assessment of the physiological effects that may be present. Nevertheless, the interest for AgNPs is

remarkable For the wide range of applications, there are many opportunities to find them in the food chain both for environmental pollution, for example, due to the release of textiles during washing (Geranio et al., 2009) and for all products sold outside the EU and can easily enter by the online market. For this reason, appropriate analytical methods are needed to extract and characterize these metallic nanoparticles from complex matrices such as food.

1.4. Study Design

At the beginning of research, a literature study was conducted to evaluate methods to extract AgNPs from the media in which they were dispersed. From this evaluation it has emerged that one of the methods proposed is the Cloud Point Extraction (CPE). Since pH is one of the parameters influencing CPE, the stability and behavior of two types of AgNPs in media characterized by different pH values has been evaluated. Comparison was made using plasmon resonance (UV-VIS) and dynamic light scattering (DLS). In addition, to obtain information on shape and size distribution, suspensions of these nanoparticles in ultrapure water were analyzed by scanning electron microscopy in SEM mode (high vacuum conditions). The collected images were selected and analyzed using the ImageJ software.

In literature CPE is described for AgNPs, but not for these nanoparticles in complex matrices such as foodstuffs. For these reason a recent scientific article describing CPE as technique to separate AgNPs from environmental water was tested for its applicability to food matrices. AgNPs were spiked in ultrapure water and in food matrices (juices and milk). After CPE, distribution of silver in the pellet and in the surfactant phases has been determined. For this purpose, exemplarily analysis method by ICP-MS after microwave digestion has been optimised for CPE with silver spiked in ultrapure water and then applied for all samples. In addition, different steps of CPE tested with AgNPs in ultrapure water have been examined by scanning electron microscope in different operation modes (Cryo-SEM, SEM and STEM), to confirm whether this extraction technique effectively preserve the shape and size of the nanoparticles.

1.5. Cloud Point Extraction: principle of the method

Cloud Point Extraction (CPE) is an alternative to conventional extraction methods , due to its lower toxicity, low cost and applicability for a wide variety of analytes; in fact, it is usually applied to pre-concentrate and extract organic substances, ionic species and nanomaterials from a medium (Duester et al., 2016).

CPE is based on the capacity of a surfactant solution to form micelles when it is heated to a certain temperature, called cloud point temperature (CPT). To obtain micelles is also important

that a surfactant is added into the sample at levels exceeding its micellar minimum concentration (CMC). In this way, increasing the temperature of 15 - 20 °C above the CPT (Nollet & Toldrà, 2015), the surfactant solution becomes cloudy and the extraction within the micellar aggregates occurs. A subsequent centrifugation and decantation allow to obtain two phases: a liquid phase (supernatant) and a surfactant rich-phase (pellet) containing the analyte of interest. The main steps of CPE are presented in Figure 3 (Samaddar and Sen, 2014).

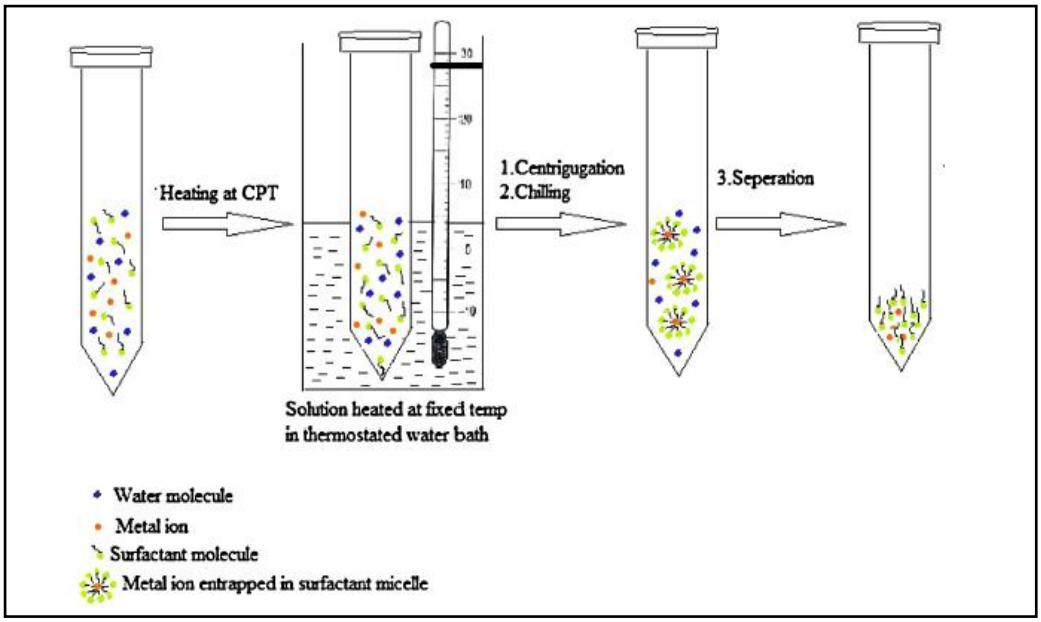

Figure 3 . Representation of cloud point extraction (Samaddar & Sen, 2014).

CPT strictly depens on the surfactant used. There are different types of surfactants (zwitterionic, cationic, anionic and non-ionic) on the basis of the hydrophilic group (Biparva & Matin, 2012), but in CPE non-ionic detergent are often investigated (see Table 2).

Table 2 . Examples of surfactants commonly used in CPE (Raynie, 2016)

Non-ionic surfactant	Cloud point temperature (°C)
Triton X-100	66
Triton X-114	25
Brij 30	2
Brij 35	> 100
Brij 56	69
Brij 97	72
Tween 80	65

Among surfactant shown in Table 2 Triton X-114 is extensively used in CPE (Paleogos et al., 2005), due to its low cost, relatively low CPT and high purity.

CPE is usually used to concentrate nanomaterials from environmental waters (Liu et al., 2009; Chao et al., 2011; Hartmann et al., 2013) or biological samples (Yu et al., 2013). Until now there are no studies involving this technique for silver nanoparticles isolation from food matrices. Therefore, it was decided to investigated whether CPE could be a promising analytical approach to concentrating and separating nanosilver from complex matrices such as food, assuming that these nanoparticles were in foodstuff due to a migration phenomenon from the packaging. For this purpose, recent scientific article (Yang et al., 2016) describing CPE as technique to separate AgNPs from environmental water was tested.

A lot of parameters have effect on the CPE such as pH, surfactant concentration, ionic strength, temperature, equilibration time and centrifugation. These factors should be evaluated to obtain the best performance from this sample pretreatment method, but it was beyond the scope of this study.

2. MATERIALS AND METHODS

2.1. Chemicals

Nitric acid (100 %, p.a) and sodium thiosulfate anhydrous (99 %, p.a) were purchased from Carl Roth (Germany). Triton X-114 (TX-114, laboratory grade) was obtained from Sigma-Aldrich, USA.

Hydrogen peroxide (30 %) and nitric acid (65 %) at high purity grade (Suprapur) used for microwave-assisted digestion were purchased from Merck GmbH (Germany), as well as citric acid monohydrate (for analysis) and tri-Sodium citrate dehydrate (for analysis).

Ultrapure water (18,2 MΩ×cm, 25 °C) for UHPLC and LC-MS was used in all the experiments (MillQ, Merck Millipore, Germany).

2.1.1. Silver nanoparticles

Two different types of silver nanoparticles suspension were used in this explorative study: AgPURE^{TM} and Citrate BioPure^{TM} silver.

AgPURE^{TM} W10 (Lot. Number A10_151102) is an aqueous dispersion of nanosilver with a nominal silver content of 10 w/w%. This dispersion has a yellow-brown colour and was purchased from RAS AG (Germany). It contains particles with a nominal diameter below 20 nm, which are stabilized with surfactant agents: polyoxyethylene glycerol trioleate (Tagat TO$^{®}$) and polyoxyethylene (20) sorbitanmonolaurate (Tween 20$^{®}$). From original product (100000 ppm) a

stock solution of 1000 ppm was prepared by dilution and kept away from light, at room temperature. Working concentrations of 50 ppm, 20 ppm, and 5 ppm were freshly prepared immediately before use. All dilutions from original product were prepared in gravimetric way, as recommended in the technical information of AgPURE^{TM}.

Citrate BioPure^{TM} silver is a nanoparticle coated with sodium citrate. In this work citrate silver nanoparticles exhibiting different sizes and silver content were investigated (Table 3).

Table 3. Citrate silver nanoparticles investigated (diameters and silver content from product certificates).

Citrate silver nanoparticle	Lot Number	Diameter by TEM (nm)	Ag silver content (mg/ml)
5 nm Citrate BioPure^{TM} silver	MGM2206	(4.6 ± 0.8)	1.00
20 nm Citrate BioPure^{TM} silver	DMW0224	(20.6 ± 3.6)	1.06
50 nm Citrate BioPure^{TM} silver	MRL1090	(51.6 ± 5.4)	1.11
200 nm Citrate BioPure^{TM} silver	CLF0225A	(193.7 ± 21.2)	1.05

They were purchased from NanoComposix (NanoComposix; Prague, Czech Republic). From original products (1000 ppm), which were stored away from light and in refrigerate conditions, working concentrations of 50 ppm and 5 ppm were freshly prepared immediately before use.

It was observed that nanoparticles tend to settle on the bottom of the container during storage. For this reason, all original products and stock solutions were placed in a bath sonicator for no more than 30 seconds prior to use. After this step, the bottles were vigorously shaken and sonicated again for the same time. It was important to obtain homogenous suspensions.

Both for AgPURE^{TM} and for Citrate BioPure^{TM}, dilutions at final concentrations of 5 ppm and 50 ppm were made in different aqueous media: ultrapure water, ultrapure water adjusted to pH 3.0 and 3.5 using diluted nitric acid, citrate buffer at pH 4.2. This pH value were in the range of tested food products. Only for AgPURE^{TM} dilutions of 20 ppm were prepared using ultrapure water and beverages.

2.2. Silver nanoparticles characterization

2.2.1. UV-Vis analysis

UV-Vis measurements were performed in triplicates using a Unicam UV1 spectrophotometer (Unicam Ltd, UK). The UV-Vis spectra of silver nanoparticles investigatedwere recorded between 300 – 600 nm in 1 nm intervals. Silver nanoparticles suspensions were prepared in different aqueous media in a concentration of 5 ppm. For measurements disposable 1,5 ml UV cuvettes (BRAND GmbH, Wertheim, Germany) were used.

2.2.2. Dynamic light scattering (DLS)

Hydrodynamic particle sizes (Z-Average, PDI) of the used silver nanoparticles were determined in different aqueous media by DLS in a concentration of 50 ppm. Analysis was performed using a Zetasizer Nano ZS (Malvern Instruments Ltd, UK) with 633-nm red laser. Malvern Instruments software (Version 7.02) was used to control and analyze all data obtained. The dispersion medium was water exhibiting a viscosity of 0.8872 cP and a refractive index of 1.330. The reference material was silver with a refractive index of 0,135 and absorption of 3,990. Measurements were carried out in disposable 70 µl micro UV cuvettes (BRAND GmbH, Wertheim, Germany) at 25°C. The Zetasizer cell was equilibrated for 120 seconds at the selected temperature prior to data acquisition.

2.2.3. Scanning electron microscopy and images analysis.

Images of silver nanoparticles (AgPURE™) contained in CPE samples performed in ultrapure water were carried out using a scanning electron microscope (Quanta 250 FEG, FEI, Eindhoven, Netherlands) equipped with Everhart-Thornley (ETD) detector. Different operation modes were employed: Cryo-SEM, High-vacuum SEM and STEM.

Cryo-SEM: 5 µl of sample were used to fill a rivet in the specimen stub, which was placed in the transfer holder. Only the sample volume from "pellet" was diluted 1:2 with ultrapure water. The sample was quickly frozen in a slushy nitrogen freezing station connected to a rotary pump, where the temperature was reduced from -196 °C to -210 °C. After the sample was directly transported into a preparation chamber (-135 °C) for subsequent processing: freeze-fracture, sublimation step (10 min, -90 °C) and platinum sputtering (30 seconds) under pure argon. Finally, the specimen was transferred to the microscope chamber (-135 °C, high vacuum) onto a nitrogen gas-cooled stage.

High vacuum-SEM: a sample volume of 6 µl was pipette onto a silicon-chip (Plano, Wetzlar, Germany) fixed on an aluminium specimen stub (Agar Scientific, UK) with special adhesive support tabs (Leit-Tabs, Plano, Wetzlar, Germany). Only the sample volume from "pellet" was diluted 1:2 with ultrapure water. After the sample was air-dried for about 30 min and finally transferred to the SEM stage for analysis.

Both for cryo-SEM mode and for high vacuum-SEM, energy-dispersive X-ray spectroscopy (EDS) (EDAX GenesisApex SM 2i, EDAX Inc., Mahwah, USA) was used to confirm the presence of AgPure™ in the samples, employing the following analytical conditions: accelerating voltage 10 kV and working distance 10 mm.

STEM (Scanning Transmission Electron Microscopy): 6 µl sample were pipetted onto a TEM grids (diameter of 3 mm, copper, 300 mesh) with holey carbon film (Plano, Wetzlar, Germany). Only the sample volume from "pellet" was diluted 1:10 with ultrapure water. After this step each grid was air-dried for about 30 min and finally transferred to the STEM stage for analysis. With the STEM detector integrated in the sample stage holder, dark field and bright field observations were possible. The working parameters employed were: accelerating voltage 30 kV, working distance 6 mm and high vacuum.

The operation mode *High-vacuum SEM* was also used to collect images for AgPure™ and citrate silver nanoparticles investigated in this study. Working concentrations in ultrapure water were freshly prepared immediately before the observation (Table 4).

Table 4. Working concentrations employed for High-vacuum SEM observation.

Silver nanoparticle	Concentration
200 nm Citrate BioPure™ silver	10 ppm
50 nm Citrate BioPure™ silver	5 ppm
20 nm Citrate BioPure™ silver 5 nm Citrate BioPure™ silver AgPURE™	2.5 ppm

The chosen images were analyzed with ImageJ image analysis software (version 1.50i) for obtaining particle size distribution.

ImageJ, an open source software, was used for particle analysis of silver nanoparticles. Original images, collected with a scanning electron microscope in SEM high vacuum condition, were in TIFF format. Image analysis process was performed as follows.

- Pixel values are converted into nm (*set scale*), using the scale placed on the SEM images and considering that 1µm is equal to 1000 nm.
- The image was converted to 8-bit greyscale.
- The scale bar was removed from the image (*crop*).
- *Auto threshold* was applied to automatically set the minimum and the maximum grey level value to discriminate between objects and background.
- The image was converted into *binary image*, consisting of pixels having value of 0 or 1 and this allows to usually have image with black objects and white background.
- Each pixel was replaced with the median value in its 3x3 neighborhood (*despeckle*).
- *Erode* and *dilate* (removes and adds pixels from the edges of objects in a binary image, respectively) were used to obtain smooth objects and removes isolated pixels.
- Touching particles were automatically separated by *watershed*.

After the selection of specific settings in ImageJ (like show outlines, display results, exclude on edges and include holes), a spreadsheet with the values of areas and a final image that reports a number for each particle were obtained.

The image analysis method and the graphical representation of result of particle size analysis were performed on the basis of ISO standards (ISO 13322-1:2014; ISO 9276-1:1998).

2.3. Determination of silver nanoparticles from water and food matrices

2.3.1. Sample preparation procedure

In this study, *cloud point extraction (CPE)* was used to separate silver nanoparticles from food matrices. The CPE procedure was carried out in accordance to a recent scientific article (Yang et al., 2016). Briefly, 10 ml of aqueous sample, 50 µl of 1 M $Na_2S_2O_3$ and 200 µl of 10% (w/w in ultrapure water) TX-114 were placed in a 15 ml centrifuge tube. Initially, the experiments were performed using a silver nanoparticles suspension in ultrapure water adjusted to pH 3.5 using diluted HNO_3. Afterwards, silver nanoparticles suspensions in food products were employed (Table 5).

Table 5. Food products used for CPE experiments. These food products were purchased in German supermarkets in Karlsruhe (Germany).

Product (original name)	Type of product
Eos Bio, Apfelsaft, Direktsaft	Clarified apple juice
Jacoby, Apfelsaft Klar, Directsaft	Clarified apple juice
Kumpf, Weiβer Traubensaft, Direktsaft	Clarified white grape juice
Hipp, Birnesaft Bio+Mineral Wasser	Pear juice+mineral water (for children after 4 month)
Weihenstephan, Haltbare Milch 0,1 % Fett	Skimmed milk 0,1 % fat

All silver nanoparticles suspensions had a concentration of 20 ppm and were prepared in gravimetric way. For CPE experiments only AgPURE™ was employed.

Samples were stirred 1 min using a vortex, incubated at 40°C in a water bath (Certomat®, WR, B. Braun Melsungen AG, Germany) for 20 min, centrifuged at 2800 rpm (~ 600 g) at room temperature for 5 min (Hettich Zentrifugen, Germany) and cooled at 4 °C for 1 hour. All experiments were performed in triplicates. The two phases obtained (surfactant-rich phase called "pellet" and the aqueous phase called "supernatant") were separated using a Pasteur pipette, weighed out in TFM™-PTFE (PTFE modified with perfluoroalkoxy chain) digestion vessels and digested with a microwave unit (Multiwave GO, Software version 1.10, Anton Paar GmbH, Austria). The EPA method 3052 was applied and subsequently modified to adapt it to the digestion procedure normally used in the laboratory. Digestion was performed by adding 4 ml

65 % nitric acid and 0.5 ml 35 % hydrogen peroxide and using the following program: heating up to 120 °C in 10 min for 10 min and then increasing up to 180 in 10 minutes for 30 minutes. After cooling the vessels, each digested sample was diluted with ultrapure water and analyzed by ICP-MS for the total silver content.

2.3.2. Analysis of silver nanoparticles by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

The silver concentration in digested samples was determined by using an inductively coupled plasma mass spectrometer (iCap Q, Thermo Fisher Scientific Inc., Dreieich, Germany) with SC4DX Autosampler (ESI Elemental Service & Instruments GmbH).

The instrumental parameters are listed in Table 6.

Table 6.Working parameters of ICP-MS.

Parameter	Value
RF power	1550 W
Argon flow rates	
- Cooling	13,8 L/min
- Auxiliary	0,65 L/min
- Nebulizer	1,05 L/min
Sample cone	Ni
Skimmer cone	Ni
Analyte	^{107}Ag
Internal standard	^{103}Rh
Aquisition/scanning mode	KED-H2
Collision cell	8% H2 in He
Sweeps per reading	100
Dwell time	10 ms
No. of replicates	3
Sampling depth	7 mm
Nebulizer pump speed rpm	40
Concentric nebulizer flow	0,4 ml/min
Chamber Temperature	2,7 °C

Silver isotope ^{107}Ag was detected. Rhodium ^{103}Rh contained in concentration of 4 ppb in a mixed standard stock solution of 100 mg/l in 2% HNO_3/tr HF was used as an internal standard (M2-IS2-100, ESI Elemental Service & Instruments GmbH, Germany).

Before ICP-MS analysis all digested samples containing 65 % HNO_3 were diluted with ultrapure water to obtain a HNO_3 concentration of 2 %.

The concentration of silver was quantified by calibration curves. They were prepared for each type of sample using the original food products and a certified silver concentration of

1.002 ± 6 µg/ml in 5% HNO_3 v/v (Inorganic™ Ventures). Calibration curves at three concentrations levels were used:

- 0,1 ppb, 1 ppb, 10 ppb, for original food products;
- 1 ppb, 10 ppb, 100 ppb, for original food products with 20 ppm of AgPURE™, for supernatants and pellets.

ICP-MS software **(**Qtegra, Version 2.8.2944.115**)** was employed for data elaboration.

3. RESULTS AND DISCUSSION

3.1. Silver nanoparticles characterization

Stability/agglomeration behavior of silver nanoparticles is dependent on pH of the surrounding medium and stabilization agents. Thus stability of 2 silver preparation stabilized electrostatically and sterically has been investigated in media characterized by different pH values, using plasmon resonance (UV-VIS) and dynamic light scattering (DLS).

Silver nanoparticles with different sizes (5 nm, 20 nm, 50 nm, 200 nm) and coated with sodium citrate were compared to AgPURE (stabilized by surfactants and having a particle diameter less than 20 nm).

The following environments have been tested: ultrapure water adjusted with diluted HNO_3 to pH 3 and pH 3.5, citrate buffer pH 4.2 and only ultrapure water, pH 7. Acidified water at pH 3.5 is the situation used in the basic experiment of CPE, while pH 3 is a condition employed in other CPE experiments found in the literature (Liu et al., 2009; Yu et al., 2013). Citrate buffer pH 4.2 and ultrapure water were employed by Burcza et al. (2015) and here used again as comparative terms to see if there could be a confirmation of the results.

3.1.1. UV-Vis analysis

Stability of AgNPs suspensions in different media was measured over a time of 1 hour. Figure 4 shows the absorption spectra recorded for AgNPs suspensions at the beginning of the measurements.

In general, AgNPs interact with light and when electrons on the metal surface are excited by a light source, they are subjected to a collective oscillation. This phenomenon is called surface plasmon resonance (SPR) and depends on several parameters such as shape and size of particles or medium properties (such as refractive index).

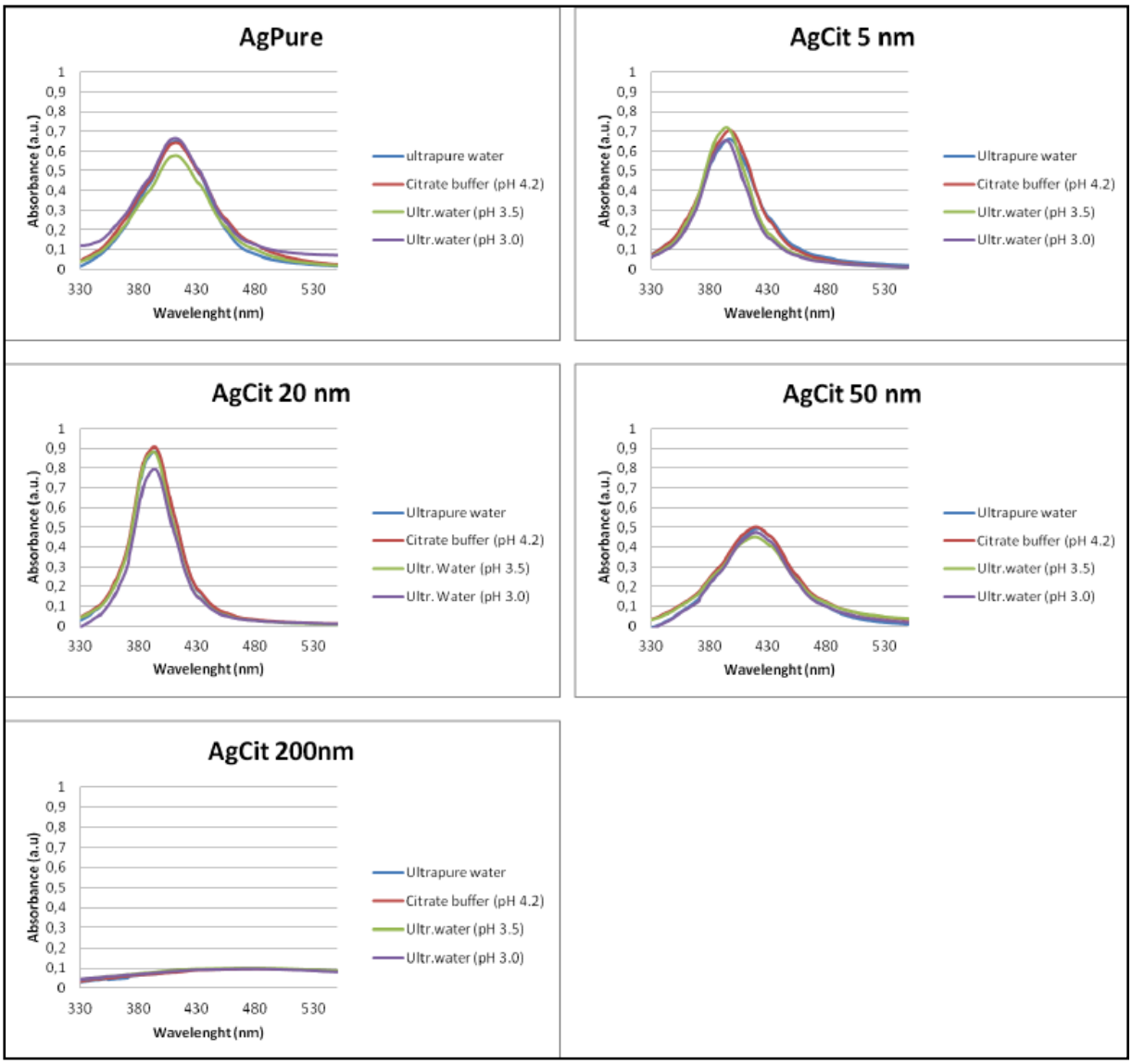

Figure 4. UV-vis absorption spectra of silver nanoparticles in different media at the beginning of the measurements. For each suspension, spectra of three independent experiments were recorded, but in the figure only one is shown.

As can be seen in Figure 4, silver nanoparticles tested, by SPR, exhibit absorption maxima at a wavelength around 400 nm. For AgCit suspension with a nanoparticle diameter of 200 nm, compared to others, there is a very different absorption spectrum. These nanoparticles have a larger size: by SPR, they show a small and broad spectrum shifted towards longer wavelengths (around 500 nm).

Absorption spectra for AgPure and AgCit suspensions in the different media were recorded over 1 h (one spectrum every 10 min) to see if there were shifts or reductions in SPR peaks. After 60 minutes (spectra not shown), no difference was observed compared to the situation at the beginning of the measurements.

Absorption spectra obtained for AgPURE in water and citrate buffer are very similar to those reported by Burcza et al. (2015), while for AgCit 20nm (in the same media) different results compared to the same scientific article has been obtained.

3.1.2. Dynamic light scattering (DLS) analysis

"DLS measures the light scattered from a laser that passes through a colloidal solution and by analyzing the modulation of the scattered light intensity as a function of time, the hydrodynamic size of particles and particle agglomerates can be determined."(NanoComposix's Guide to Dinamic Light Scattering Measurement and Analysis, 2015).

The measured size is called "hydrodynamic diameter" and is defined as the diameter of a sphere that diffuses at the same speed as the measured particle. Since analysis is performed on samples in suspension, the size obtained is not only related to the "core" of the particles, but considers the solvation sphere surrounding them.

The thickness of the solvation sphere affects the diffusion rate and depends on the adsorbed substances on the surface and the characteristics of the medium (such as the ionic concentration).

Figure 5 shows the average values of the hydrodynamic diameter (z-Average) for the AgNPs suspensions in different media.

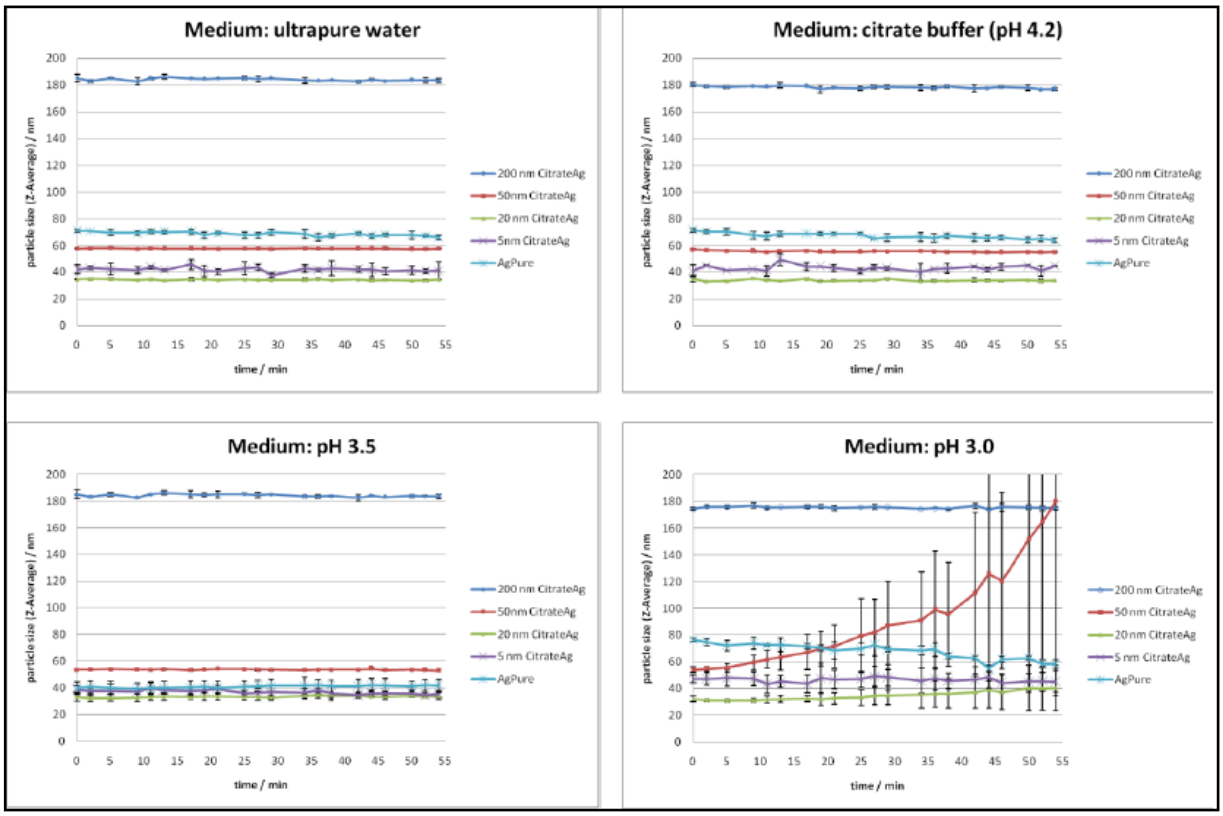

Figure 5. Z-Average values for AgNPs suspensions in different media. Analysis were performed in triplicate, over a time of 1 hour.

As shown in Figure 5, for ultrapure water, citrate buffer and water adjusted to pH 3.5 (with HNO_3), the hydrodynamic diameter of the different nanoparticles is constant over the time of analysis considered (1 hour). Although AgPure and AgCit 20 nm have comparable diameters by TEM, these silver nanoparticles show different z-Average values, probably due to the different stabilizers present on their surface.

Medium at pH 3 seems to have a lot of influence on z-Average values, especially for AgPure and AgCit 50 nm. The decreasing trend found for AgPURE may indicate a dissolution of nanoparticles. In addition, it could be possible that the "extension/conformation" of the surfactant layer around the silver core changes due to the pH value, leading to hydrodynamic diameter modifications

With regard to AgCit 50 nm there is an increase in z-Average value over time and the large error bars associated with this sample indicate that the recorded measurements are different to each other. In fact, considering the size distribution report by intensity obtained from a DLS measurement and based upon the intensity of light scattered by particles, it is possible to see what is shown in Figure 6.

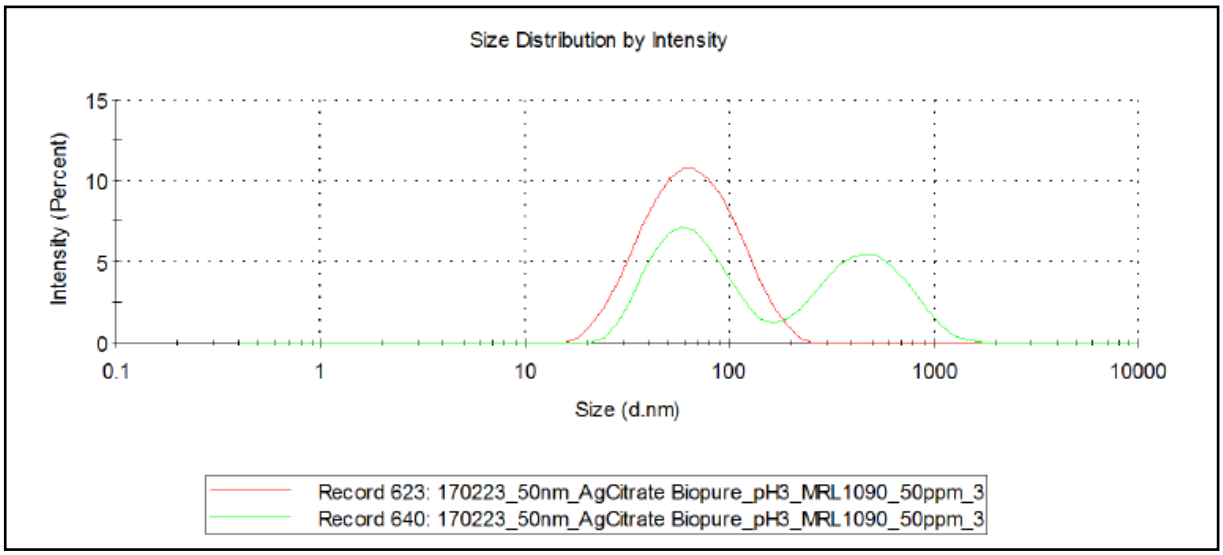

Figure 6. Example of size distribution report by intensity for two records of AgCit 50 nm in pH 3 medium.

As can be seen in the figure above, some DLS measurements related to AgCit 50 nm show a monomodal distribution (red profile) and others a bimodal distribution (green profile) indicating a non-homogeneity of the sample. The green profile in Figure 6 may depend on the fact that nanoparticles agglomeration occurs. Another hypothesis to justify the green profile may concern the accidental presence of dust (imperceptible to visual inspection) inside the cuvette used.

Figure 7 shows the polydispersity index (PDI) over time, another important parameter obtained from the DLS measurements.

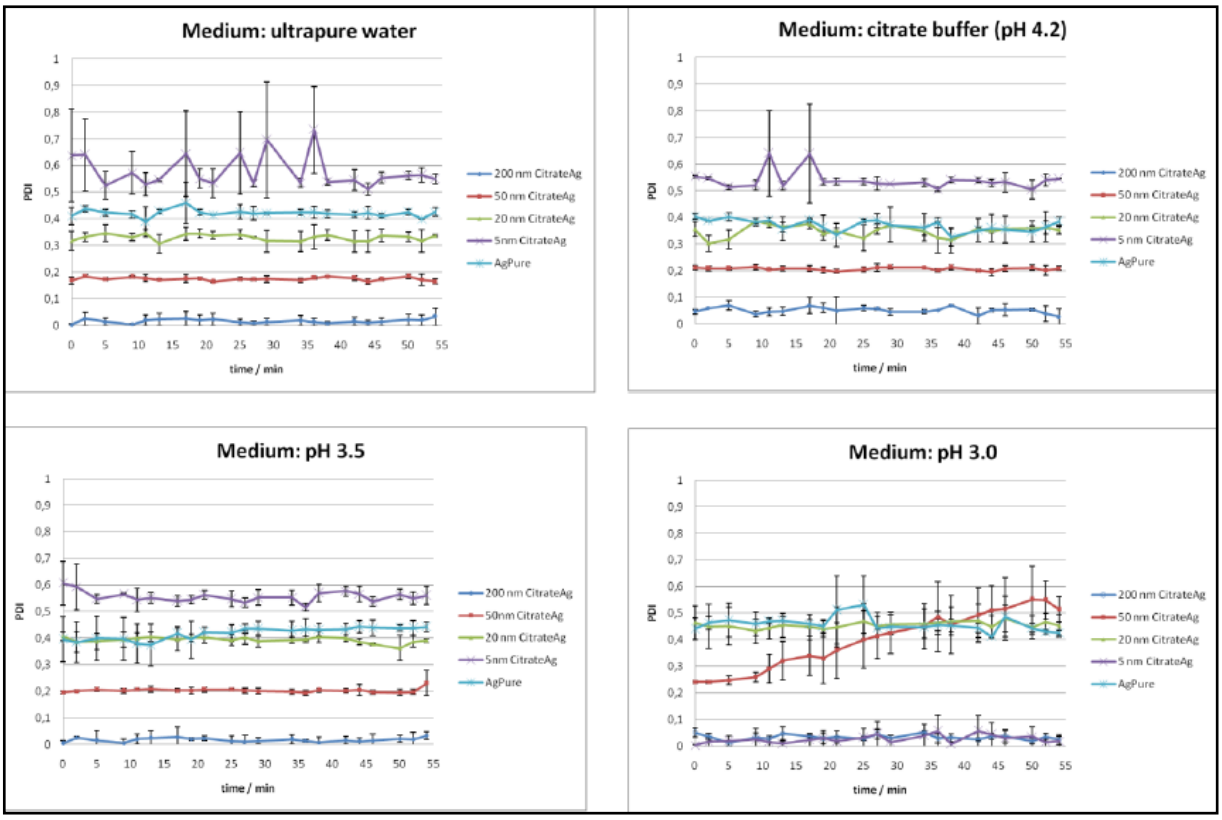

Figure 7. PDI values for AgNPs suspensions in different media. Analysis were conducted in triplicate, over a time of 1 hour.

PDI is the index of the broadness of the size distribution curve. The PDI ranges from 0 to 1: values closer to 0 representing narrow distributions, while values closer to 1 represent broad distributions. As shown in Figure 7, for different AgNPs suspensions tested, there are no PDI values > 0.7 indicating samples with a very broad distribution and probably not suitable for analysis by DLS (Malvern 2011, Dynamic Light Scattering- Inform White Paper). Trend of PDI values over time (1 hour) and in different tested media, in many cases it is similar to that observed for z-Average values (see Figure 5). In the case of DLS measurements of AgCit 5nm, the analysis often did not meet the software quality criteria. Furthemore, in the technical data sheet of this AgNPs there is no hydrodynamic diameter value specified, indicating the same difficulties to measure this nanoparticles by DLS.

AgCit 5nm can be detected by DLS, but the measured size is not correct. To justify this, it is only possible to speculate that for these small sizes the relation between particle core size and size of the hydrodynamic shell is inappropriate (large shell and small core size gives measured sizes larger than the real size). It can also be presumed that these small nanoparticles exhibit an high diffusivity which gives a bad signal/noise ratio. Finally, the small amount of scattered light may result in bad signal/noise ratios too.

3.1.3. Particle analysis by Scanning Electron Microscopy

Scanning electron microscopy operating in High-vacuum SEM mode was used to investigate size and shape of silver nanoparticles employed in this research project.

AgPure and AgCit suspensions were prepared in ultrapure water according to the "*Materials and Methods*" section.

The representative images for each nanosilver sample are shown in Figures 8-12, where it can be observed that nanoparticles are more or less spherical. The collected images were subsequently processed by Image J software to obtain size distribution graphs (see figure 9-12). For this purpose, more than 1000 particles were counted from images of each sample. Only for AgCit 5 nm it was not possible to get the size distribution graph. In fact, as shown in Figure 8, the small particles were aggregated/agglomerated and could not be clearly distinguished in order to have a correct images elaboration.

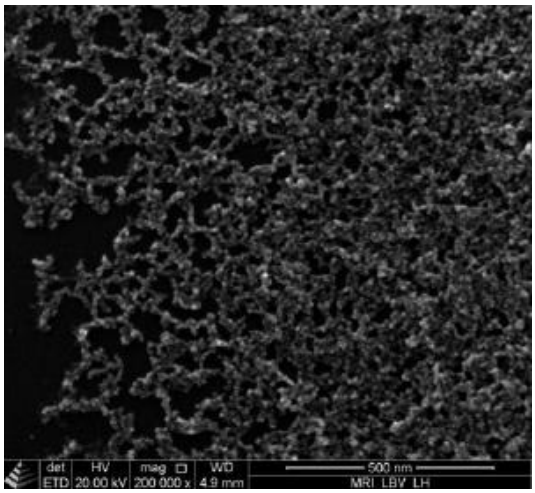

Figure 8. Representative SEM image of AgCit 5nm.

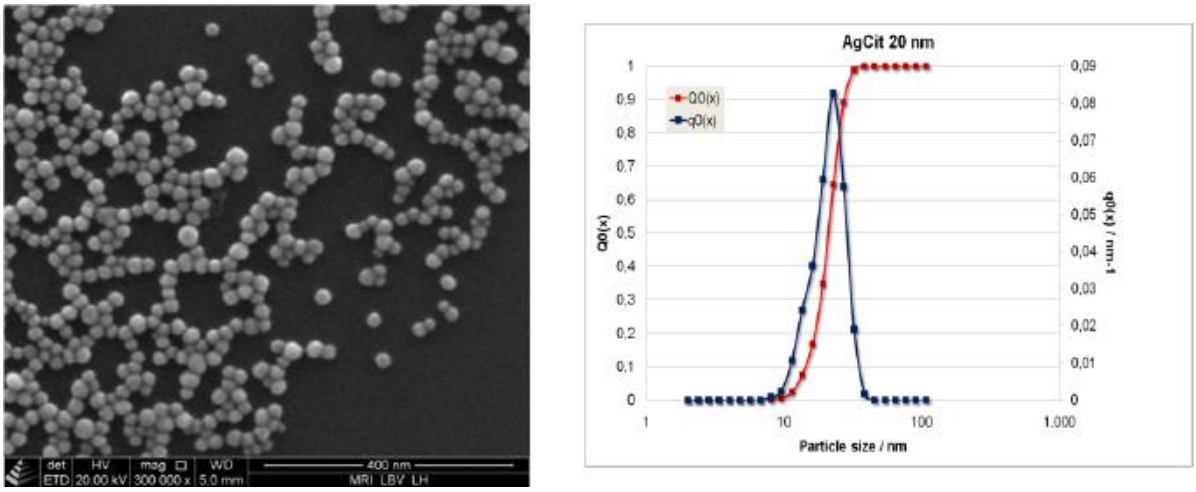

Figure 9. Representative SEM image of AgCit 20 nm (left) and related particle size distribution graph (right). Number of particles analyzed= 1981. Average particle diameter = 20.7 ± 4.8.

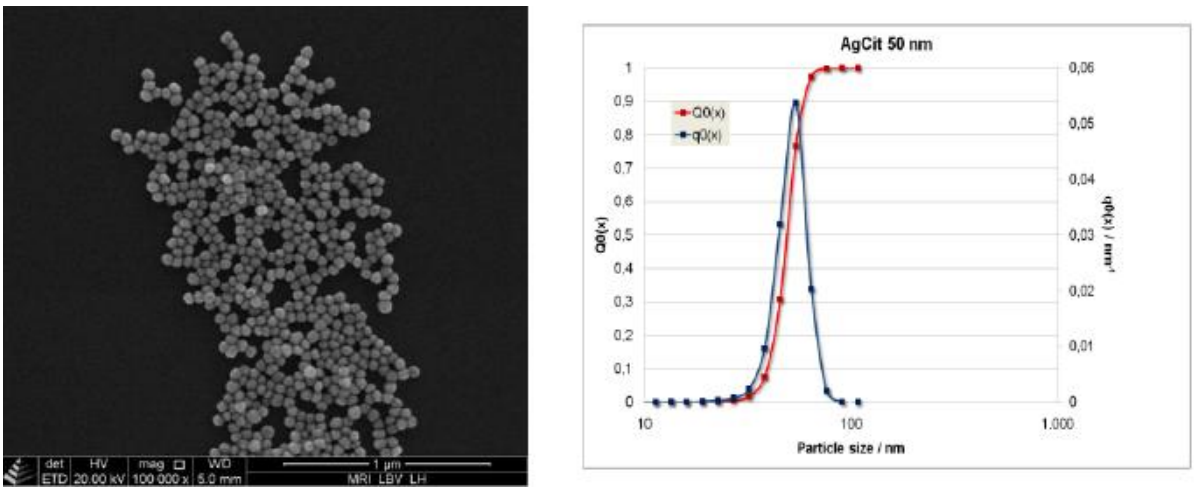

Figure 10. Representative SEM image of AgCit 50 nm (left) and related particle size distribution graph (right). Number of particles analyzed = 3228. Average particle diameter = 48.7 ± 7.6.

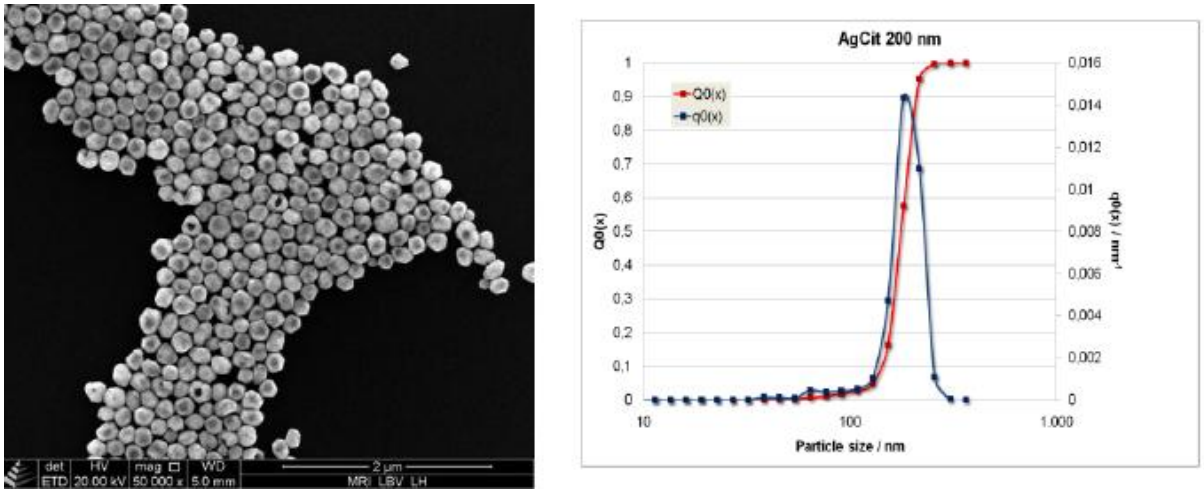

Figure 11. Representative SEM image of AgCit 200 nm (left) and related particle size distribution graph (right). Number of particles analyzed = 1295. Average particle diameter = 174.4 ± 28.4.

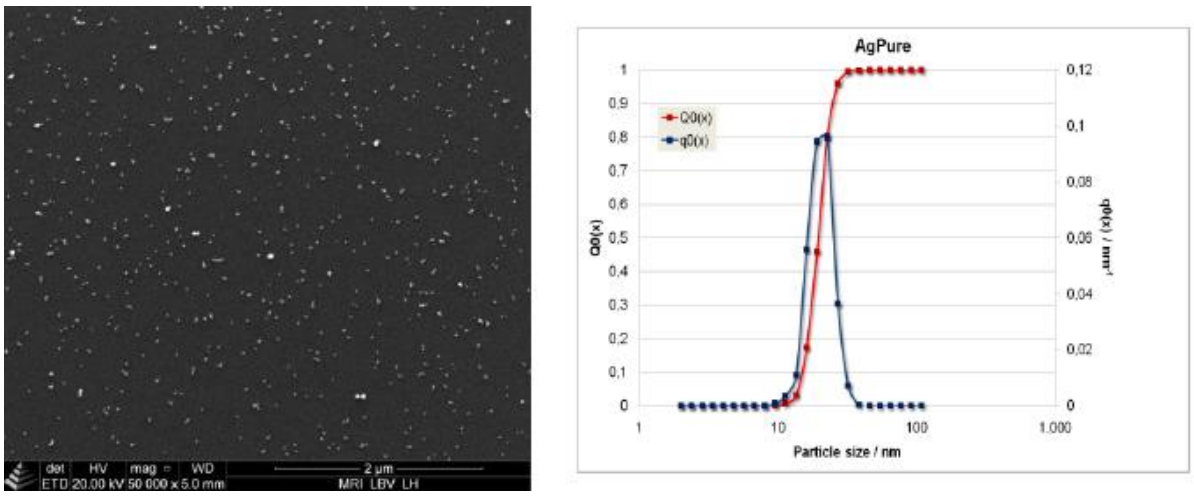

Figure 12. Representative SEM image of AgPure (left) and related particle size distribution graph (right). Number of particles analyzed = 1437. Average particle diameter = 19.6 ± 3.8.

To avoid misinterpretation of the images, it was decided not to consider the particles at the edges of the image and overlapping particles since, the latter especially, were considered as a single aggregate by the software.

As shown in Figures 9-12, there are two curves in the size distribution graphs. The red curve is related to the *cumulative size distribution Q0(x)* where each point in the distribution defines the quantity of particles with a dimension smaller than or equal to any diameter value present on the

abscissa. The blue curve is related to the *density size distribution q0(x)*, which generally makes it possible to see if the sample particles follow a normal distribution (gaussian). The differential distribution shows the relative amount of particles at each size. However, both distributions are related: integration of the differential distribution curve results in the cumulative distribution

It is important to emphasize that compared to the DLS analysis where the measured dimension is the "hydrodynamic diameter" that takes into account not only the "core" but also the solvation sphere around each particle in solution, employing the microscope the size obtained is the diameter of the particles pertinent only to the "core". In fact, the observed aliquot is a dried suspension and therefore particles do not exhibit the solvation sphere.

3.2. Determination of silver nanoparticles from water and food matrix

3.2.1. CPE in ultrapure water and analysis by scanning electron microscopy

The CPE procedure employed is in accordance to a recent scientific article (Yang et al., 2016), while AgPURE™ W10 was the silver nanoparticle investigated in this part of project. Initially CPE was tested with AgPURE dispersed in ultrapure water, to confirm whether this extraction technique effectively preserve the shape and size of the nanoparticles as reported in literature (Liu et al., 2009; Majedi et al., 2012). For this reason different steps of CPE (see Figures 13 and 14) have been examined by scanning electron microscope in different operation modes (Cryo-SEM, SEM and STEM).

In all cases it has been impossible to monitor the individual silver nanoparticles and clearly see the micelles probably due to the complexity of the samples and instrumental limits. Nevertheless, some of the collected images have led to very interesting reflections.

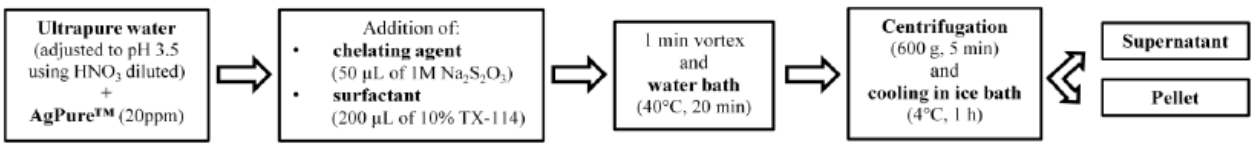

Figure 13. Diagram of cloud point extraction steps performed with AgPure in ultrapure water.

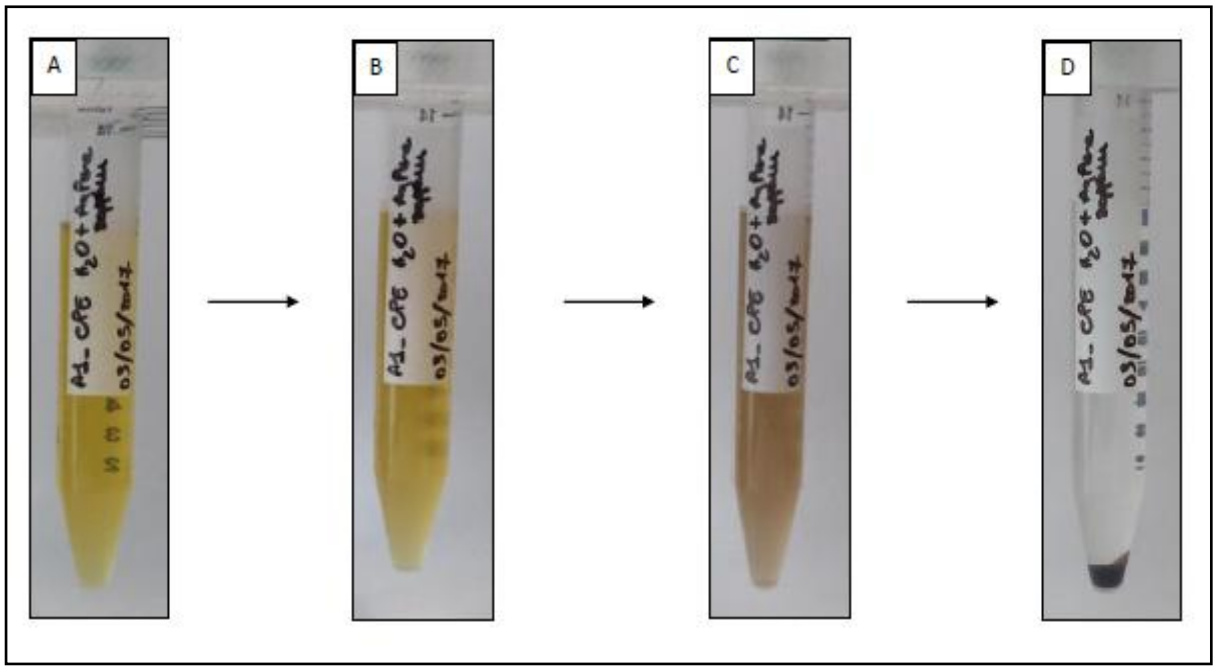

Figure 14. CPE steps performed in laboratory with AgPURE in ultrapure water.

Figure 14 shows a real CPE experiment in ultrapure water. As described in *Materials and Methods* section (point 2.3.1), 10 mL of AgPURE suspension (20 ppm) has been added in ultrapure water adjusted to pH 3.5 using diluted HNO_3 (**A**). The suspension becomes a little bit cloudy due to the addition of 50 µL of $Na_2S_2O_3$ (1 M) and especially of 200 µl of the surfactant TX-114 (10% w/w). This phase has been called "before water bath" (**B**). In third step, the suspension is cloudy and has changed colour, because it was incubated at 40 °C for 20 min (higher than cloud temperature of the surfactant). This phase has been called “after water bath” (**C**). Finally, a liquid phase called “supernatant” (upper) and a surfactant-rich phase called “pellet” (lower) have been obtained after centrifugation (~ 600 g at room temperature for 5 min) and cooling in ice bath (4 °C for 1 h) (**D**).

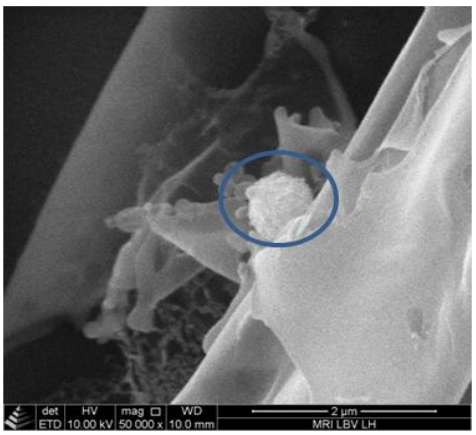

Figure 15. Cryo-SEM image of the CPE step called “before water bath”.

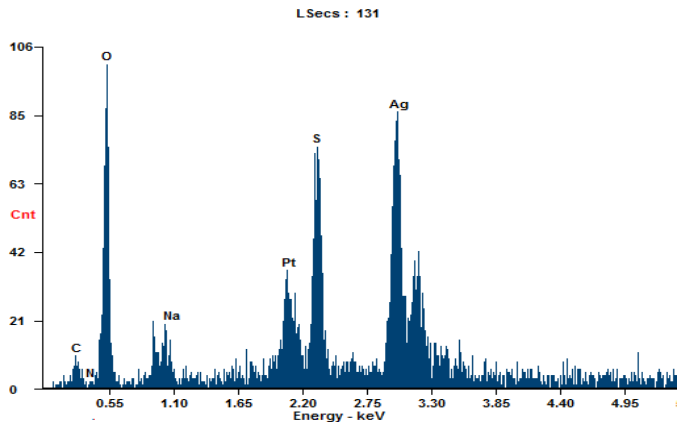

Figure 16. EDX spectrum of the detail highlighted in figure 15.

Figure 15 shows a Cryo-SEM image obtained by analyzing the sample before the thermostated bath. In the blue circle there is a spherical formation and EDX spectrum (see figure 16) at this level confirms the presence of AgNPs (peak Ag) and other chemicals needed for CPE. In fact, the high intensity of the peak O in the EDX spectrum is related to the oxygen that is part of the chemical structure both the surfactant Triton-114 together with carbon (peak C) and the salt $Na_2S_2O_3$ together with sodium and sulphur (peak Na and S, respectively). Sodium thiosulfate is used as a complex agent for Ag^+ ions released by silver nanoparticles. The peak Pt is due to platinum, employed in sample preparation for microscopic observation (see section *Materials and Methods*, point 2.2.3)

Cryo-SEM images of the sample related to the step "after water bath" (see Figure 17) and concerning pellet (see Figure 18) did not provide the expected information. After 20 minutes of incubation at 40 °C, having exceeded the CPT of the surfactant (for Triton-114 is 23 °C), the expected micelles containing AgNPs, were not found in the pellet.

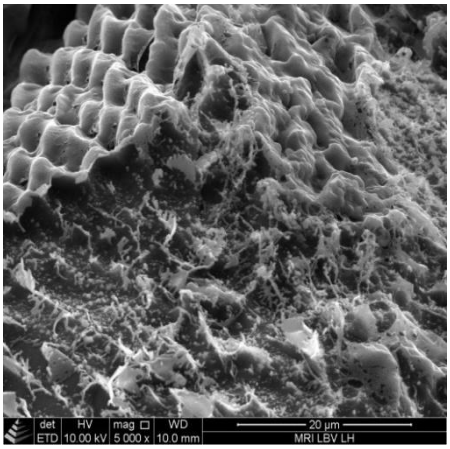

Figure 17. Cryo-SEM image of the CPE step "after water bath".

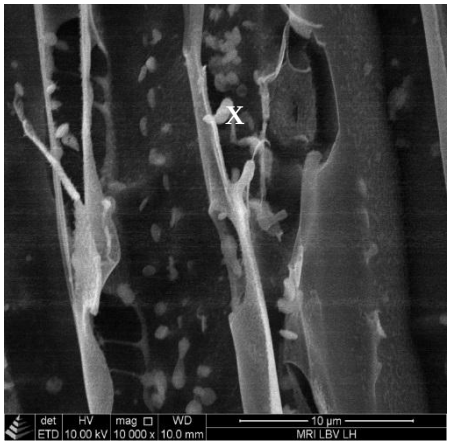

Figure 18. Cryo-SEM image of pellet obtained after centrifugation.

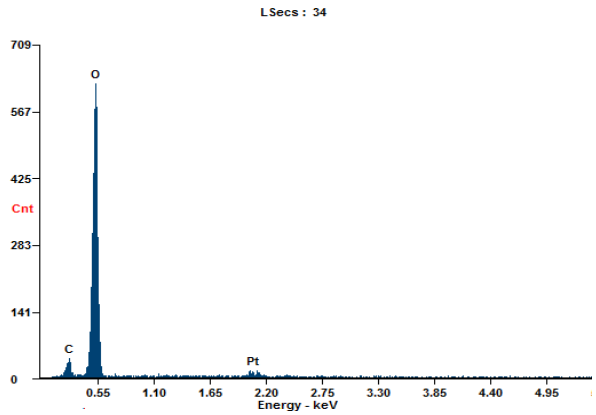

Figure 19. EDX spectrum of the detail highlighted in figure 18.

As shown in figures 17 and 18, images were difficult to interpretate, perhaps because the sample aliquot was not liquid but had to be frozen by liquid nitrogen (see section *Materials and Methods*, point 2.2.3). Probably freezing changed the sample structure, making it impossible to see and identify what was of interest.

Employing electron microscopy in SEM mode and analyzing a sample aliquot before the thermostated bath (see Figure 20), a situation similar to the one shown in Figure 15 and confirmed by EDX spectrum (see Figure 21), was observed.

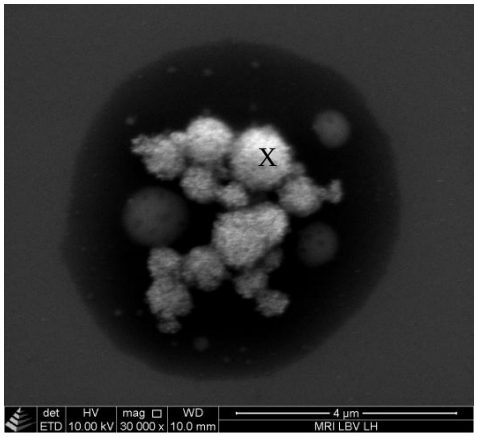

Figure 20. SEM image of the CPE step "before water bath".

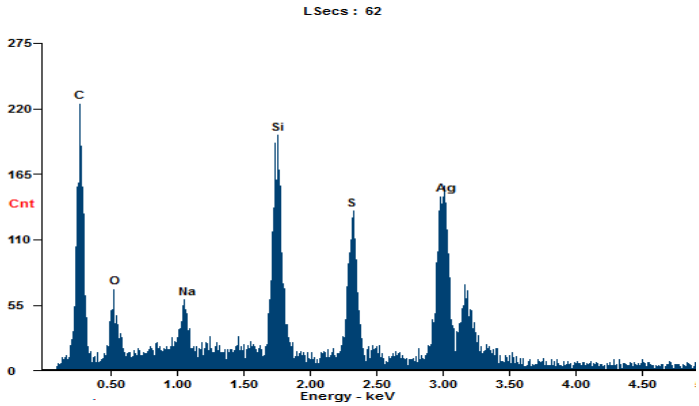

Figure 21. EDX spectrum of the detail highlighted in figure 20.

In the EDX spectrum shown in Figure 21, peak SI is due to the silicon present in the support employed in the sample preparation for microscopic observation (see section *Materials and Methods*, point 2.2.3). "Spongy spherical structures" observed (see figure 15 and 20) and their composition (see figure 16 and 21) suggest that a formation "like micelles" takes place before the increase of the temperature above the cloud point temperature (CPT), but this hypothesis was not found in literature.

Compared to Cryo-SEM analysis, images collected in SEM mode have confirmed the presence of the AgNPs even in the final steps of the CPE (see Figures 22-26), although in the EDX spectrum of pellet the peak relative to the nanoparticles (see Figures 25 and 26) is very low, contrary to what was expected.

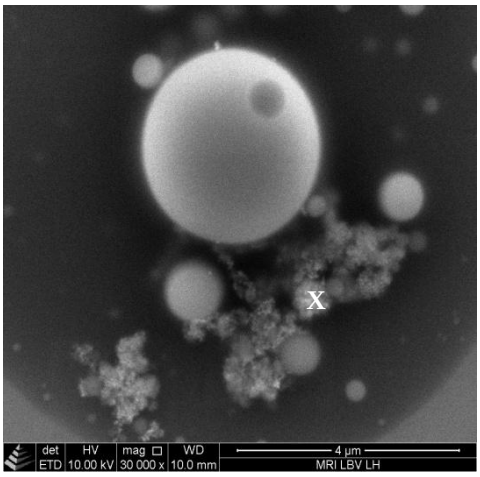

Figure 22. SEM image of the CPE step "after water bath".

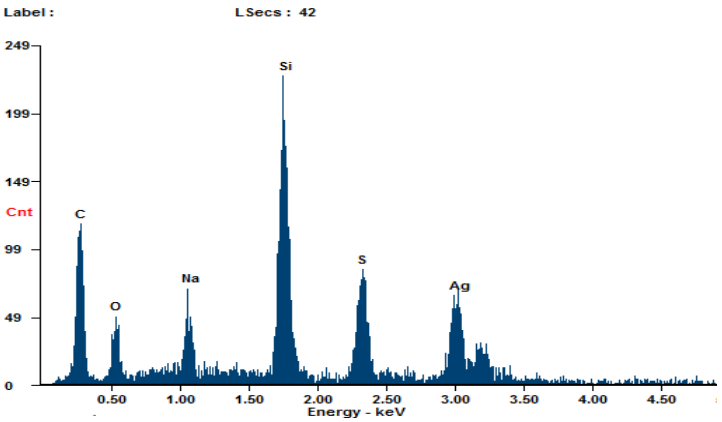

Figure 23. EDX spectrum of the detail highlighted in figure 22.

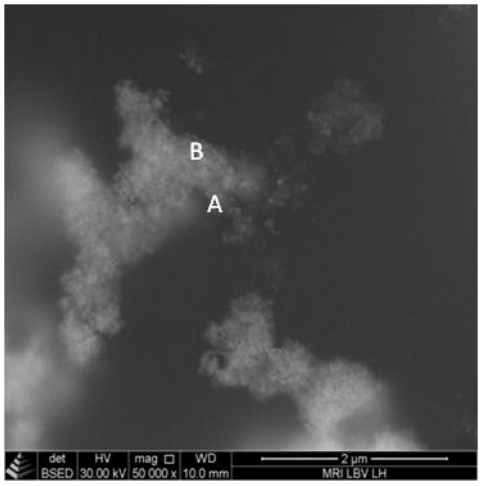

Figure 24. SEM image of pellet, obtained employing BSED detector (backscattered electron detector).

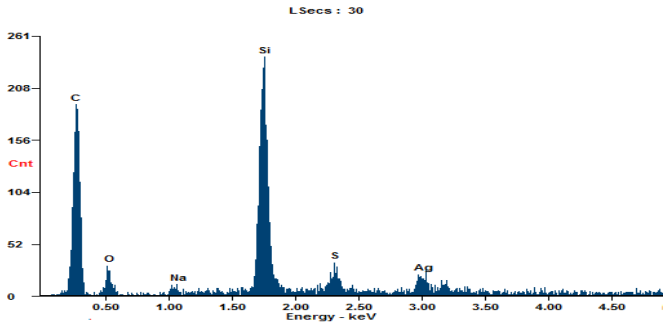

Figure 25. EDX spectrum of the area "A" highlighted in figure 24.

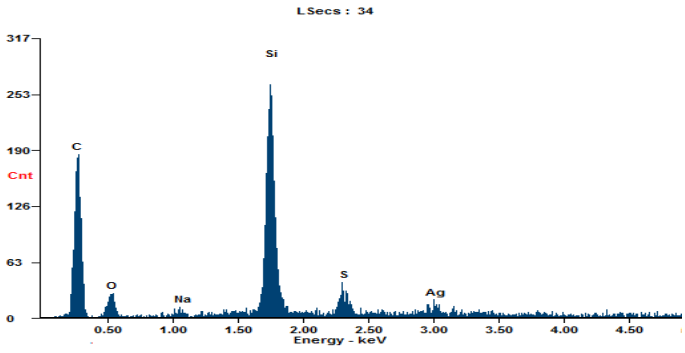

Figure 26. EDX spectrum of the area "B" highlighted in figure 24.

In Figure 22 very regular and large globular formations were evidenced, one in particular. EDX spectrum (see figure 28) performed on similar formations found in the pellet obtained without AgNPs (see figure 27), indicates the presence of peaks related to the elemental composition of salt $Na_2S_2O_3$.

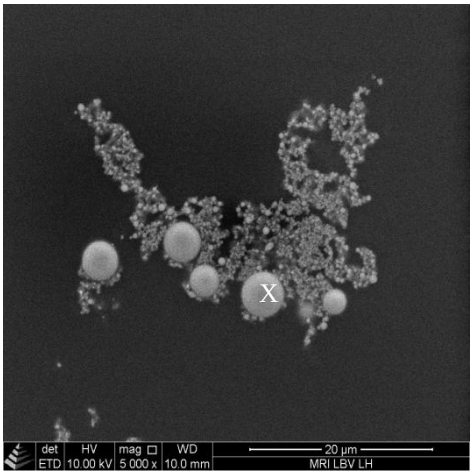

Figure 27. SEM image of pellet without AgNPs

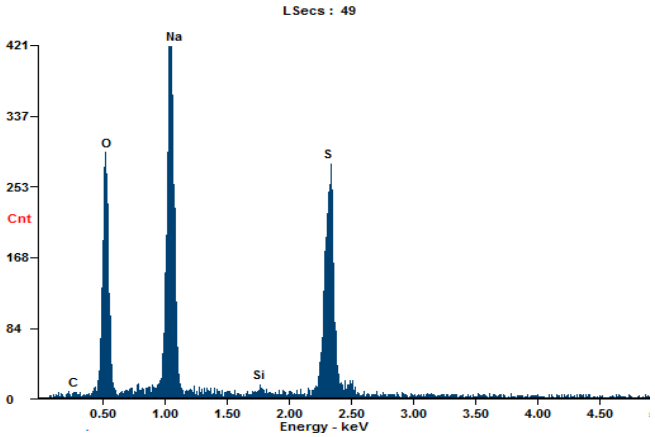

Figure 28. EDX spectrum of the detail highlighted in figure 27.

Sodium thiosulfate is used in CPE as a chelating agent of Ag^+ ions released by nanoparticles. In solution thiosulfate and silver ions form a stable complex which being negatively charged, is not included in the pellet and remains in the aqueous phase (Yang et al., 2016). Based on this and considering the presence of AgNPs in the sample, after the water bath and in the pellet, there should be present only micelles containing nanoparticles. Perhaps the presence of salt in the final stages of CPE and confirmed by EDX spectra may be due to the fact that the aliquot observed after the water bath was taken from the sample prior to the centrifugation and therefore before obtaining the two phases (supernatant and pellet). Otherwise, it may be that traces of salt at level of pellet are justified by the fact that complete removing of supernatant was difficult.

Images collected in STEM mode did not provide any further information about CPE steps. In fact, due to the particular support employed for sample preparation (see Figure 29), images were very complex (see Figure 30). In addition to the previous operating modes, the specimens were located inside the STEM detector. This prevented the use of the Dispersive X-Ray detector to obtain EDX spectra and to have information about the elemental composition of the samples.

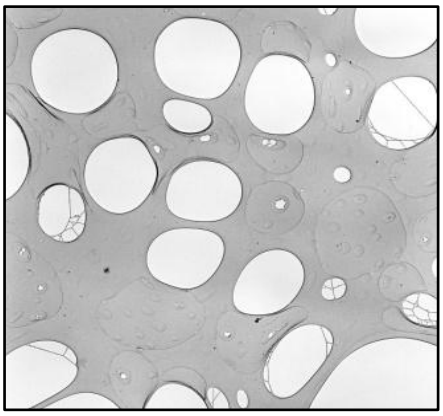

Figure 29. Holey carbon film (Plano, Wetzlar, Germany) employed.

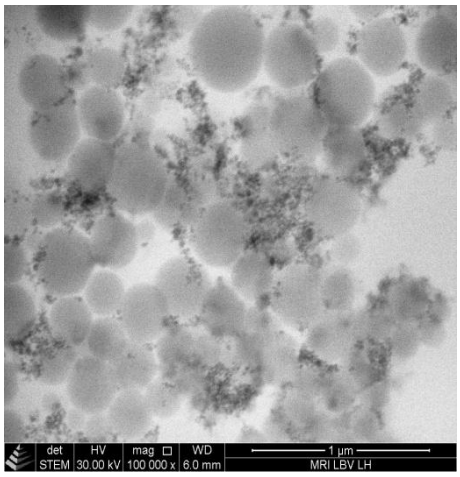

Figure 30. STEM image of pellet.

3.2.2. CPE in food matrices and analysis by ICP-MS

Difficulty in monitoring by SEM the behavior of silver nanoparticles in the CPE steps has led to the need to investigate whether AgNPs were completely extracted in the final pellet. Applying the extraction procedure described in the section *Materials and methods* CPE was carried out spiking AgNPs both in ultrapure water, and using food matrices such as fruit juices (apple, grape, pear) and skimmed milk (0.1 % fat). These food matrices have been used without pH correction. pH values of different beverages are shown in Table 7.

Table 7. pH values of food matrices investigated.

Sample	pH value
Eos Bio (filtered apple juice)	3.51 ± 0.01
Jacoby (filtered apple juice)	3.44 ± 0.01
Hipp (pear juice+mineral water)	4.03 ± 0.02
Kumpf (filtered white grape juice)	3.45 ± 0.02
Weihenstephan (skimmed milk 0,1 % fat)	6.59 ± 0.02

The total silver content in the samples was determined in triplicate by ICP-MS after microwave digestion of the pellet and supernatant of each sample (see Table 8).

Table 8. Ag content determined by ICP-MS in 10 g of sample

Sample	Nanosilver content (Ag/µg)	
	Pellet	**Supernatant**
Ultrapure water	177.75 ± 3.62	41.00 ± 2.13
Eos Bio (filtered apple juice)	155.02 ± 7.62	23.49 ± 1.76
Jacoby (filtered apple juice)	145.16 ± 5.47	26.97 ± 7.07
Hipp (pear juice+mineral water)	107.05 ± 4.93	39.32 ± 10.34
Kumpf (filtered white grape juice)	42.56 ± 1.38	17.51 ± 15.46
Weihenstephan (skimmed milk 0,1 % fat)	13.71 ± 1.88	1.19 ± 0.10

In the case of an ideal situation, Ag would have to be found only in the pellet, instead from results obtained and shown in Table 8, it is possible to understand that CPE is not exhaustive because small amounts of silver are found in the aqueous phase.

Microwave digestion has been applied before ICP-MS to destroy all organic compounds in samples. However, even after digestion some matrix compound is still present, leading to a possible matrix effect; this could be improved by method development of the digestion process for each sample.

An important question is the possibility to measure silver amount into sample after digestion and dilution directly in ICP-MS, or the necessary to adopt a standard addition method. This investigation has been done for CPE experiment performed in ultrapure water. It has been found that in case of the supernatant it was possible to measure the supernatant after digestion and dilution directly in ICP-MS. Instead, the pellet was spiked with different known concentrations of silver standard (see *Materials and Methods* section) because the CPE chemicals (surfactant, in particular) influenced the ICP-MS signal, even after microwave digestion and dilution. The calibration plot obtained after ICP-MS measurement of pellet, has been used to calculate silver

amount of unknown samples. Despite this, the interferences have only been corrected and not completely eliminated.

If different samples are examined, this procedure is time consuming and beyond the scope of the present study. For this reason method development has only been done for CPE in ultrapure water and then applied for CPE in juices and milk too.

As shown in Table 8 good results have been obtained for silver amount found in the pellets of all samples, except for grape juice and milk. It is possible that the amount of sugars in the first case (grape juice) or the casein micelles in the second case (milk) interfere with the extraction technique. This matrix effect may also be due to an incomplete digestion of the organic component. Finally, another effect (more physical) could be responsible for the bad recoveries. Microwave digestion has been used to destroy organic material, but also to dissolve the silver nanoparticles to silver ions. Ag^+ are very reactive and could form insoluble compounds for example with chlorides or sulphur which precipitate and therefore are not captured by the ICP-MS. If this effect exists, it should be established or developed a special procedure to dissolve the precipitate.

4. CONCLUSIONS

Stability of two silver nanoparticles (AgPure and AgCit) used in the present study was found to be highly related to the medium and stabilisation agents.

It is known that surface plasmon resonance (SPR) depends on several parameters, among which surface properties around the particles. Thus, if two samples are stabilized by different surfactants, it could be presumed that differences in SPR occur too.

Regarding the quality of DLS analysis, it has been confirmed the same difficulty of the manufacturer to measure very small nanoparticles (for example AgCit 5nm). To justify this, it has been supposed that for particles with small sizes the relation between particle core size and size of the hydrodynamic shell is inappropriate (large shell and small core size may give rise to measured sizes larger than the real size).

The applicability of Cloud Point Extraction (CPE) for pre-concentration and separation of silver nanoparticles from complex matrices such as food, has been tested. The CPE protocol used was in accordance to Yang and co-workers (Yang et al., 2016), which applied this technique to extract AgNPs from environmental waters. CPE was performed spiking a commercially available nanosilver dispersion (AgPure) before in ultrapure water and then in food products (juices and milk). Analysis of total silver content by ICP-MS after microwave digestion, revealed that AgPure is not completely isolated in the pellet. Therefore, CPE steps should be optimized on the

basis of the different parameters affecting extraction (pH, surfactant concentration, ionic strength, temperature, equilibration time and centrifugation).

Furthemore, method development for microvawe digestion for each samples could be improved to destroy all organic compounds. Since after digestion process some matrix compound may persist, a method development is necessary.

Regarding microscope analysis, it could be interesting to investigate CPE steps (not only in ultrapure water but also in food products) by transmission electron microscopy (TEM). This technique compared to scanning electron microscopy (SEM) provide high-resolution images, allowing to obtain morphology data of sample. Nevertheless, sample preparation could be difficult (especially for food matrices), due to the need to have very thin specimens for TEM analysis.

Finally, the use of food matrices would have been better also for evaluate the stability/behaviour of AgNPs suspensions. Unfortunately, these investigations by DLS or UV-vis are difficult and often impossible, due, for example, to the absorption of the medium and the complexity of food matrices.

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CHAPTER 4: UHPLC-HRMS FOR DETECTING MIGRATING COMPOUNDS FROM FOOD CONTACT MATERIALS

1. INTRODUCTION

1.1. Food contact materials

We define "food contact materials" all materials and articles intended to come into contact with foodstuffs, from the most common such as packaging and containers, kitchen utensils, cutlery and crockery, to those belonging from the industrial components of the machinery used in their production, processing, transportation, etc.

These materials can be made of plastics, rubber, paper, glass, metal and others. The European Community legislation governing the materials in contact with food in the legislative apparatus also including those in contact with water intended for human consumption, such as bottles, with the exception of the fixed water supply, both public and private.

The safety of the materials intended to come into contact with food should be subject to evaluation and control because of the possibility of accidental migration of chemicals contained in their composition or the formation of new compounds, directly into foodstuff. The materials must be manufactured according to precise rules laid down by the Community regulations, including good manufacturing practices, so that any potential transfer of substances to foods does not result in a hazard to the health or an unacceptable change in the composition or sensory quality of the product.

In defense of consumers and to protect the safety of food produced and marketed, materials and articles intended to come into contact with food are governed by both National and Community measures.

With regard to the Community framework under the EC Regulation 1935/2004 (REGULATION (EC) No 1935/2004), it establishes the general requirements to be met by all materials and objects in question, and the specific directives contain detailed provisions for individual materials (plastics, ceramics, etc..). Good manufacturing practice for materials and articles intended to come into contact with food are described in the EC Regulation 2023/2006 (COMMISSION REGULATION (EC) No 2023/2006).

The Decree of the Italian Minister of Health of 21 March 1973, the following materials have been governed: plastics, rubber, regenerated cellulose, paper and cardboard, glass and stainless steel (Decree of the Minister of Health of 21 March 1973).

This decree has been amended several times to comply with the provisions of the European Union.

The spirit of the legislation is based on the "positive list" of substances that can be used in the production of such materials by any limitations and restrictions, with the aim to check the suitability for food contact (Decree of the Minister of Health of 21 March 1973).

Considering their undoubted importance and diffusion, the use of plastics in the food industry is also regulated by a specific Directive (Commission Directive 2002/72/EC), which sets out a list of authorized monomers, other substances and additives authorized for the manufacturing of plastic material, as well as the overall and specific migration limits.

Over time, this Directive has been amended and revised several times in order to adapt to constant technological advances in the field of food production, and for this reason, the European Commission decided to establish a new regulation to replace part of the old directive. In 2011, EU legislation related to plastics used in food contact materials has been consolidated into a single instrument, the *EU Regulation 10/2011* called "PIM" (Plastic Implementation Measure) published in the Official Journal of the EU (Gazette No 12 of January 14, 2011) consisting of 23 items, divided into 6 chapters and 6 annexes (COMMISSION REGULATION (EU) No 10/2011).

This regulation includes a list of substances authorized for the production of plastic materials in contact with food and establishes their overall and specific migration limits.

Overall migration is defined as the total quantity of substances released by the material into food simulant; the specific migration, is the quantification of the individual substances migrated from the material to the food.

The migration assays are performed using food simulants, i.e. substances or mixtures of them with high affinity to the food for which the plastic article is intended.

These limits are based on the assumption of a daily exposure for the entire life of a person of 60 kg to 1 kg of food products packed in plastic packaging containing the substance in the maximum amount permitted and are as follows:

- Overall Migration Limit (OML): set at 10mg of substances/dm² of surface area of the materials in contact with the food product for all substances that can migrate into food. In some cases the overall migration limit is expressed in 60 mg/kg of food;
- Specific Migration Limit (SML) for individual substances authorized, established on the basis of a toxicological evaluation, and assuming standard exposure.

Compliance with the limits of migration lies is a responsibility of the manufacturer of plastic materials, which must direct the consumer or the food manufacturer to the most appropriate use of the object, through the drafting of a Material Safety Data Sheet (MSDS) in which also these information are declared.

Data related to this chapter have been reported in the following publications:

- Bignardi C., Cavazza A., Laganà C., Salvadeo P., Corradini C., (2015). *UHPLC-High Resolution Mass Spectrometry determination of bisphenol A and plastic additives released by polycarbonate tableware: influence of ageing and surface damage*. Anal Bioanal Chem, 407, 7917-7924;
- Bignardi C., Cavazza A., Laganà C., Salvadeo P., Corradini C., (2017) *Release of non-intentionally added substances (NIAS) from food contact polycarbonate: Effect of ageing.* Food Control, 71, 329-335
- Bignardi C., Cavazza A., Laganà C., Salvadeo P., Corradini C., *Optimization of mass spectrometry acquisition parameters for determination of polycarbonate additives, degradation products, and colorants migrating from food contact materials to chocolate*. Journal of Mass Spectrometry, in press.

1.2. Polycarbonate: an example of plastic material for food contact

Polycarbonate (PC) is a widely used material for food contact, and it is often employed for tableware such as plates and cups construction, and for food storage containers aimed also at reheating the product in microwave oven.

Stability of plastic material during ageing has been studied, and age was reported to be responsible for changes in many properties of an amorphous material that undergo structural changes with time becoming more and more glass-like (Struik, 1977). Even exposure to light can induce structural modification in the polymer (Collin et al., 2012). It is possible to distinguish between chemical and physical ageing, although usually the two processes concur to a global effect. The combination of many factors such as temperature, exposure to ultraviolet and visible light, atmospheric components, humidity, or contact with liquids, can affects plastic stability (Harvey, 2012). PC, in particular, has been found to be very susceptible to water attack, determining the carbonate linkage to be split into an alcohol with the release of carbon dioxide (Factor, 1996). Besides, contact with methanol (Kim et al., 2009; Liu et al., 2009) and amine groups (Maia et al., 2010; Van Delinder et al., 2015) has been found to increase its degradation.

Physical damage can also cause PC breakage and formation of tiny scratches or small cracks on its surface that becomes opaque and foggy with a consequent release of polymer components.

The main concerns about degradation of PC employed as food contact material is linked to the release of its monomer, bisphenol A (BPA), originating both from disruption of the polymer, and from unreacted monomers (Hoekstra & Simoneau, 2013).

BPA has been extensively studied for its effects on human health (Rochester, 2013). Many researches have been performed concerning BPA quantification in food simulants (Alin & Hakkarainen, 2012; Fasano et al., 2012) and in real food samples, and regarding factors affecting its release (Hoekstra & Simoneau, 2013), also from reusable food containers such as baby bottles (Biles et al, 1997).

The main factors affecting BPA release are time of contact, temperature and physico-chemical properties of the simulant/food, depending on its pH and composition (Hoekstra & Simoneau, 2013). Some authors found a linkage between amount of BPA released and possible content of amine (Maia et al., 2010) or ethanol (Biles et al, 1997) and the type of water employed, in terms of pH but also of mineral composition (Biedermann-Brem & Grob, 2009; Oca et al., 2014). A study showed a clear dependence of BPA release on thermal treatment and effect of microwaves (Biedermann-Brem & Grob, 2009).

An important point that it is worth to be considered, regards the re-usable plastic containers aimed at storing food products, since they are submitted not only to ageing process, but also to mechanical damage due to the repeated use and frequent washing cycles. The integrity of the surface is a critical factor; in fact a high release of bisphenol A can be expected after damage concurring during cleaning procedures such as dishwashing, sterilization and brushing (Brede et al., 2003). For instance, the employment of basic detergents has been found responsible of a large BPA amount release (Maia et al., 2009) due to its capacity of catalyzing PC hydrolysis (Mercea, 2009).

Differences between BPA release by new and used baby bottles have also been evaluated (Kawamura et al., 1998; Tan & Mustafa, 2003; Le et al., 2008; Nam et al., 2010).

It has never been investigated whether the BPA release is more affected by the ageing of the material, regardless its use, or by the physical damage of the surface due to repeated use.

In order to protect the polymer and prevent BPA release, additives such as antioxidants and UV absorbers are often employed in PC construction. Obviously, the additives themselves may also represent possible migrants into the food. Several studies reported qualitative and quantitative determinations of some additives, mostly assessed by GC or HPLC coupled to UV or fluorescence detector (Alin & Hakkarainen, 2012; Sanches Silva et al., 2006; Gao et al., 2001).

In this context, the establishment of the identity of all substances released by plastic, and the evaluation of the main parameters affecting their migration (time of contact, temperature, type of food, etc.) is of paramount importance (Hoekstra & Simoneau, 2013). This matter does not regard only food, but involves the pharmaceutical and cosmetic fields too, since those products are often packaged in plastic, and many medical devices are made of plastic.

In addition, it is worth to consider that the process of migration is not only related to storage and packaging, but also to industrial processing and manufacturing; in fact industrial plants often contain plastic parts, and contact at high temperature and for long time may occur.

In addition to classical molecules that can potentially migrate from plastics, there is a number of potential toxicant residues known as emerging contaminants that can be defined as unregulated. Some of them have been recently discovered and are of potential concern for human health, such as degradation products derived from plastic polymers and residual monomers or oligomers not chemically bound to the polymer molecules and free to move within the polymer matrix. They can be grouped under the name of non-intentionally added substances (NIAS). The identification of these molecules, together with the estimation of a potential threshold effect for risk assessment is a relevant issue that deserves great attention in terms of safety concern (Brüschweiler, 2014; Hollnagel et al, 2014; Ketelslegers, 2014).

In this context, the development of innovative analytical methods based on the employment of modern techniques such as high resolution mass spectrometry (HR-MS) has been demonstrated to be very useful and effective for the identification of unknown compounds released by plastics used in food contact materials (Canellas et al., 2015a; Canellas et al., 2015b; Bignardi et al., 2014).

The utilization of mass spectrometry for testing food products with high accuracy and throughput has grown rapidly, and it is now recognized as an extremely specific and exceptionally sensitive tool (Pico, 2015). HRMS analyses are largely driven by the advantages of using the full scan acquisition mode with high sensitivity combined with high resolution power (> 30000 FWHM) and accurate mass measurement (1-5 ppm).

Until now several studies have been reported application of the hybrid instrument Q-Exactive in the field of food quality and safety (Zhao & Jiang, 2015; Guo et al., 2015; Li et al., 2016; Li et al., 2017; Bignardi et al., 2017). However, in literature there are no data about extraction methods of most used plastic additives and BPA from complex food.

Goal

The goal of the present work was to develop a quantitative method by UHPLC-ESI-Orbitrap on the basis of a previous qualitative approach (Bignardi et al., 2014), in order to evaluate the release of BPA and additives from food containers of different age and different degree of physical damage, and to explore a possible correlation between the investigated parameters.

The attention was focused on the untargeted analysis of simulants put in contact with re-usable objects made of polycarbonate (PC) allowed for food contact, and in particular with the aim of

identifying molecules derived from a possible degradation of the material during ageing. UHPLC coupled to HR-MS has been applied for the identification of oligomers. The study has been carried out by employing two different food simulants. Previous studies reported the identification of some molecules deriving by PC degradation after ageing and thermal decomposition of PC (Jang & Wilkie, 2004; Collin et al., 2012), nevertheless, no literature dealing with the possible migration of such substances in simulants and/or in food products can be actually found. Similarly, there are no studies about possible migration of colouring agents from tableware, although this topic should be of great interest in the safety assessment (Council of Europe, 1989).

In this work the ability of quadrupole orbitrap mass spectrometer was exploited for the first time for the quantification and determination of most used plastic additives also in a very complex matrix choosing chocolate as a model for a food with a high fat content. A full factorial and a d-optimal experimental design were employed for optimization of orbitrap acquisition parameters in order to improve sensibility. The optimized system was also applied to the detection of degradation products and colorants.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals were of analytical reagent grade. Methanol and water used as eluents were of UHPLC–MS grade and were purchased by Sigma Aldrich (Milan, Italy). Ammonium formate used as additive in eluents, ethanol and isooctane employed as simulants were also purchased by Sigma Aldrich (Milan, Italy). Pierce LTQ Velos ESI Positive ion and Pierce LTQ Velos ESI Negative ion calibration solutions from Thermo Fisher Scientific (Rockford, IL, USA) were used to calibrate the mass spectrometer. Standards of Bisphenol A, 2-(2-hydroxy-5-tert-octylphenyl) benzotriazole (Cyasorb UV5411), 2-(2-hydroxy-3,5-di-tert-butylphenyl)-5-chlorob-enzotriazole (Tinuvin 327), 2-(2H-benzotriazol-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol (Tinuvin 234), 2,5-bis(5-tert-butyl-benzoxazol-2-yl)thiophene (Uvitex OB) were purchased from Sigma Aldrich (Milan, Italy).

n-Hexane, diethyl ether, acetonitrile HPLC grade used for sample preparation were purchased by Sigma-Aldrich (Milan, Italy).

2.2. Samples

Fourteen PC tableware samples of different age and color were collected from the same supplier and were produced by the same company. Samples were named accordingly to year of production

(between 1996 and 2013) and color. Three of them (made in 2013) were never been used. The internal surface area of contact with food was calculated for each sample. Ethanol 95% (v/v) and isooctane were selected as simulants for migration experiments, and the volume of solvent needed to fill each container was evaluated.

Migration tests were carried out in compliance with the requirements of the EU Regulation 10/2011 on plastic materials and articles intended to come into contact with food. Briefly, containers filled with simulant were placed in a climatic chamber at 40 °C for 1 h (repeating the experiments three consecutive times) covering the sample with glass plates to prevent solvent evaporation. Each aliquot obtained after each migration test was evaporated by rotary evaporator and re-dissolved in 1 mL of ethanol for LC analysis. Migration values were expressed in µg Kg^{-1} on the basis of a surface/volume ratio of 6 dm^2 per Kg of food simulant according to European Commission (Regulation (EC) No. 10/2011).

The degree of damage of the PC has been evaluated by examination of the surface by a lens (10X) and a digital microscope. To each sample, a label corresponding to numbers from 1 to 5 was assigned according to the amount of scratches or cracks occurring on its surface. Label 1 was assigned to new and unused samples, characterized by a perfectly clean surface, while higher numbers were assigned progressively to more damaged surfaces (Bignardi et al., 2015).

Dark chocolate was obtained by a local factory, avoiding any technological process involving contact with plastic materials, and stored in aluminum containers. Before experiments chocolate was melt at temperature of 30 °C in a glass container, put in contact with tableware, and placed in a climatic chamber (ventilation 100%, humidity 65%) for 1 h at 40 °C. Each experiment was repeated three times consecutively on each sample, in accordance to the Regulation regarding reusable containers (Regulation (EC) No. 10/2011).

Extraction procedure (food matrix)

1 g of chocolate was placed in a glass centrifuge tube and added to the mixture of the selected standards (10 ng of each substance). Sample was dissolved adding 5 mL of diethyl ether under magnetic stirring for 10 minutes. Then, the tube was placed in the centrifuge at 4000 rpm, for 10 minutes, at 4°C. After centrifugation, the supernatant was removed and collected in a 50 mL flask. The procedure was repeated with 5 mL of diethyl ether; the supernatants were reunited and evaporated by rotary evaporator. The oily residue obtained was submitted to three steps of liquid-liquid extraction with acetonitrile (5mL x 3). Supernatants were removed with a pasteur pipette and placed in a 50 mL clean flask and evaporated by rotary evaporator, and resuspended with 2

mL of acetonitrile. The extract was loaded on a C18 cartridge and the analytes were eluted by using 10 mL of ACN (x 2).

All experiments were performed in duplicate and in parallel to a procedure of "preparative blank", simulating all extraction steps with solvent and standards, in absence of the sample, in order to identify any contaminants from the tools and the solvents employed, or deriving by the instrument.

2.3. UHPLC-HRMS Analysis

Analyses were performed by UHPLC-HRMS employing a Thermo Scientific Ultimate 3000 RSLC nano system operating in capillary-flow mode coupled to a Q Exactive Mass spectrometer (Thermo Scientific, Fremont, CA).

Chromatographic separation was carried out on a C18 Acclaim PepMap RSLC (Thermo Scientific, Fremont, CA) column (150 mm × 0.3 mm, 2.0 µm particle size) thermostated at 35 °C. Solutions employed as eluents were: 1 mM ammonium formate in 10:90 methanol:water v/v (eluent A), and methanol containing 1 mM ammonium formate (eluent B). Solvent B was initially set at 40% and then delivered by a linear gradient to 99% in 20 min at a flow-rate of 10 µL min^{-1}. Solvent B was maintained at 99% for 20 min before column re-equilibration (10 min). Injection volume was 1 µL. Q-Exactive instrument was equipped with a pneumatically assisted ESI interface with a stainless steel needle adapted for capillary flow. Conditions of the interface were fixed according to a previous reported method. (Bignardi et al., 2014). Mass calibration of orbitrap was performed every three days in order to obtain a mass accuracy lower than 2 ppm. Chromeleon 6.8 and XCalibur 2.2 softwares (Thermo Fisher Scientific MA, USA) were used to control the instrument and for data processing.

Signal acquisition was performed by full-MS-data dependent MS/MS experiments with inclusion list in the range 90-1000 m/z, in both positive and negative mode. The extraction from full MS of each parent ion taken into account allowed the visualization of the isotopic pattern and the matching between the exact mass and the accurate mass. MS/MS spectra obtained in the same run were used for confirmatory purpose (Bignardi et al., 2015).

Data-dependent acquisition of collision-induced dissociation fragmentation mass spectra was initiated automatically using an inclusion list that included information on retention times (*tR*), *m/z* values and optimal normalized collision energies (NCE) of analytes. The quadrupole isolation window was set to 4 Da. Dynamic exclusion was set off and loop count=8.

Untargeted analysis was performed through data dependent acquisition mode, without using inclusion list. The interpretation of each single fragmentation spectrum was necessary to attribute

an identity to a unknown precursor ion. Starting from the accurate mass value reported by the full scan or the fragmentation spectrum, the used software allowed to generate a list of molecular formulas (with a maximum mass deviation of ±2 ppm) suggesting potential results and giving the possibility to verify the isotopic ratios matching.

2.3.1. Quantitative analysis and method validation (simulants)

For quantitative purpose, Full MS-data dependent MSMS experiments with inclusion list were exploited. In this case, the selected resolving power influences the scan speed, which is essential to the number of data points produced for the peak of each analyte. When more scan events are added at the same time the scan speed decreases. Therefore, a compromise between the advantages of the high resolving power and the produced accuracy is necessary. Good peak shapes and mass assignment over the entire peak of the analytes were observed at R = 35 K for Full MS, by switching between positive and negative polarity, since the data points were adequate with an average number of 10, without showing mass deviations in the applied mass extraction window (±10 ppm). Moreover, dynamic exclusion was off and automatic gain control was set at 1×10^6. The extraction of molecular ion from Full MS gives the possibility to verify the isotopic pattern and the matching between the exact mass and the accurate mass of each compound. The MS/MS spectra obtained in the same run were used for confirmatory purpose. This kind of acquisition guarantees several levels of identification of a compound by matching of the accurate mass and the isotopic pattern, retention times and fragmentation spectrum.

Validation of the whole analytical methods were performed according to Eurachem guidelines (EURACHEM Guide, 1998) in terms of limit of detection (LOD), limit of quantification (LOQ), linearity and accuracy (precision and trueness).

LOD and LOQ values were calculated from the calibration curve (built in ethanol 95% v/v) as 3 *s*/slope and 10 *s*/slope, respectively, where *s* is the standard deviation of the blank signal obtained from ten independent blank measurements.

After variance homogeneity assessment ($p>0.05$) and testing significance of the intercept ($p<0.05$), linearity was also mathematically verified by applying the Mandel fitting test ($p>0.01$). Quantitative analysis was performed using the calibration curve built in ethanol 95% (v/v) by using the external standard method. Calibration curves were built by injecting in triplicate standard solutions, prepared at five concentration levels, in the range reported in Table 1.

Accuracy was assessed in terms of precision and trueness. Precision was assessed as RSD% in terms of intra-day repeatability and intermediate precision (inter-day repeatability) of retention times and peak areas. For this purpose, the within-day repeatability was evaluated by performing

three independent treatments of each simulant sample fortified with standard solution, and three injections for each sample in the same day. The inter-day repeatability was calculated on five days by performing three independent treatments and three injections per day for each sample.
Trueness was evaluated on two concentration values (LOQ x 10 and LOQ x 20), different from calibration levels, as percent ratio between calculated and spiked standard concentrations. All measurements were repeated three times.

2.3.2. Quantitative analysis and method validation (chocolate)

Validation of the entire analytical procedure was performed under MS mode either by FullMSddMSMS and tSIMddMSMS on fortified samples of chocolate according to Eurachem guidelines (EURACHEM Guide, 1998). For this purpose, chocolate samples were fortified with different amounts of standard mixture and measurements were carried out by monitoring the parent ions. Quantitative analysis was performed using the calibration curve built in matrix by external standard method. The matrix-matched calibration curve was obtained by analyzing the matrix extracts fortified with a mixture of the five standard at six concentration levels in duplicate and treated applying the entire analytical procedure. Precision was evaluated as RSD% for each compound in terms of intra-day repeatability and intermediate precision (inter-day repeatability) on two concentration levels (LOQ and intermediate level over the calibration range).
Intra-day repeatability was assessed by performing three independent extraction replicates at each level in the same day, whereas intermediate precision was estimated over five days by performing independent extractions; for each extract three LC-MS/MS injections were carried out. Trueness was calculated in terms of percent recovery as a ratio of determined and added std content on matrix at two concentration levels (LOQ and intermediate calibration level). The recovery was evaluated by performing extraction on the blank matrix and on the matrix fortified at two different concentration levels (10 and 20 ng of each standard/g). For quantitative purpose, two different acquisition methods FullMS-ddMSMS with inclusion list and tSIM-ddMSMS were exploited. Data-dependent MSMS spectra of each analyte recorded in matrix were used for confirmatory purpose

2.4. Data analysis

Means, standard deviations (SDs) and correlation values were calculated with SPSS (version 19.0, SPSS Inc., Chicago, Illinois, USA) statistical software.
3D plots of degradation products areas monitored were obtained with the Statgraphics Centurion 16.1 software.

The coefficients of the polynomial model for each standard were calculated using the R-based chemometric software developed by the Group of Chemometrics of the Division of Analytical Chemistry of the Italian Chemometric Society.

3. RESULTS AND DISCUSSION

3.1. Bisphenol A and plastic additives released by polycarbonate tableware

Since the degradation of the polymer is significantly related to its intrinsic composition (for example a PC with high degree of polymerization would be much more stable than a low degree of polymerization PC), all the experiments were conducted on similar materials made by the same producer.

Migration tests were carried out by employing two different food simulants, ethanol 95% (v/v) and isooctane, selected on the basis of literature studies (Hoekstra & Simoneau, 2013). Results obtained with the two solvents showed similar profiles from a qualitative points of view. Apart from the PC monomer bisphenol A, migrating in significant amount from all samples, some other compounds were identified in most samples. In particular, three common additives employed as UV absorbers, Cyasorb UV5411, Tinuvin 327, Tinuvin 234, and the whitening agent Uvitex OB, were found. Larger amounts for all molecules were detected when employing ethanol 95% (v/v). For each compound of interest, the two most representative transitions were selected and monitored on the basis of a previous study (Bignardi et al., 2014). A chromatogram showing the peaks detected, is reported in Figure 1.

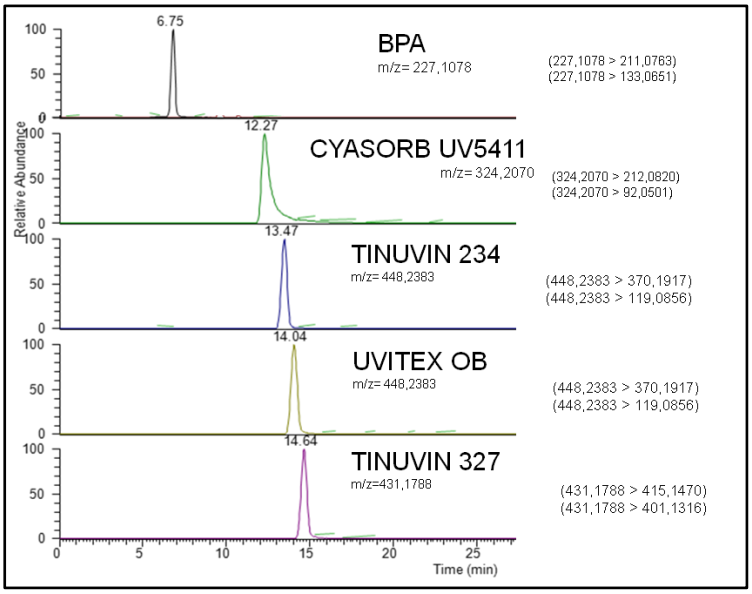

Figure 1. Chromatographic peaks of the additives investigated.

Method validation for quantitative purposes was conducted according to Eurachem guidelines (EURACHEM Guide, 1998), as reported in Materials and Methods. Obtained data are shown in Table 1.

Table 1. Data of method validation (ng mL^{-1}).

Analyte	LOD, LOQ	Linearity range	Calibration curve	r^2	Trueness %	Intra-day repeatability (area, ret time)	Inter-day repeatability (area, ret time)
Bisphenol A	0.75, 2.5	2.5-500	y=(66±0.5)x10^3x	0.9994	98±1.8	≤ 3.0%, ≤ 2.8%	≤ 3.1%, ≤ 3.0%
Tinuvin 327	0.60, 2.0	2.5-500	y=(1900±7.1)x10^3x	0.9998	90±1.5	≤ 2.9%, ≤ 2.8%	≤ 3.0%, ≤ 2.9%
Cyasorb UV5411	0.30, 1.0	1-100	y=(5444±14)x10^3x-(295±66)x10^4	0.9999	92±1.1	≤ 3.0%, ≤ 2.9%	≤ 3.0%, ≤ 3.0%
Tinuvin 234	0.03, 0.1	0.1-100	y=(1744±26)x10^4x	0.9981	98±8.2	≤ 3.1%, ≤ 2.8%	≤ 3.0%, ≤ 2.9%
Uvitex OB	0.03, 0.1	1-100	y=(1297±23)x10^4x	0.9922	95±1.2	≤ 3.0%, ≤ 2.8%	≤ 3.1%, ≤ 3.0%

In a first step, migration experiments were carried out on five samples of different age, color and shape, by employing the two selected solvents, ethanol 95% and isooctane, in order to select the most effective one for the study. From the comparison between the amounts of BPA released in the two simulants, it was seen that ethanol always extracted higher amounts than isooctane for all samples examined. In the three consecutive extractions performed, a progressive decrease of the

analyte was recorded almost in all experiments, although, data from literature (Elhert et al., 2008) reported in many cases no correlation between BPA released and consecutive migration extracts.

Experiments on the fourteen samples object of the study were then performed by employing ethanol 95% (v/v). All values were below the specific migration limit of 0.6 mg Kg^{-1} imposed by the plastic regulation (Regulation (EC) No. 10/2011). From the data obtained it can be observed that the highest levels of BPA have been found in old samples. Therefore it can be hypothesized that the small amounts released from new samples derives from unreacted monomer, while the more abundant amount found in old samples may come from polymer degradation.
No data about the frequency of use of each sample were provided, therefore in order to investigate if BPA release was linked simply to the sample ageing or to a physical damage due to the repeated use, an evaluation of the surface status was carried out. To this aim, a classification of the samples through an examination of the sample surface by a binocular lens was performed. A number from 1 to 5 corresponding to an increasing level of damage, was attributed to each sample as follows:

1 – new samples, perfectly clean surface

2 – used samples, presence of light scratches

3 – used samples, presence of many scratches and few cracks, visible only by lens

4 – used samples, presence of cracks evident even without lens

5 – used samples, presence of many cracks evident even without lens.

The lowest level was assigned to all unused samples, as their surface was perfectly integer. Oldest samples received the higher level of damage probably since they have been more subjected to chemical and mechanical stress. However, the level of damage was not always parallel to the age, since it depended on the frequency and modality of employ, and would also be correlated to washing conditions and temperature cycles. Therefore, this assigned degree could be considered as a way to experimentally estimate the level of damage of the samples.

Data were elaborated with the aim of evaluate possible relations between the variables considered: released BPA, ageing (years) and damage level linked to scratches and cracks occurrence.

It was seen that aged and damaged samples shown a higher BPA release than new samples. By calculating the pairwise correlation, the following values were obtained:

BPA - Age = 0.862; BPA – Damage = 0.786; Damage - Age = 0.863

These values confirm that correlation of BPA with age is higher than that with damage level.

These data could lead to hypothesize that aged samples, even unused, may undergo to a progressive deterioration possibly due to simple light exposure, and could become degraded regardless the frequency of use. Further studies are encouraged to evaluate such hypothesis.

Release of additives

All the detected additives were approved by FDA or the European Legislation to be employed as additives in food contact plastic (Regulation (EC) No. 10/2011).
Regarding the amount released in the two solvents employed, a behavior similar to that seen for BPA was observed. All additives were found to migrate in higher amounts in 95% ethanol. The recorded values were always decreasing from the first to the third extraction step. The decrease was steeper in ethanol than in isooctane; in fact isoctane showed always higher amount than ethanol in the third extraction. This behavior demonstrated that ethanol was more exhaustive (effective) than isooctane, while a slower kinetic extraction is followed in isooctane.

The amounts of additives recorded was found to progressively decrease during consecutive migration steps. In all cases, the amounts of additives were all below the limits reported by legislation. No correlations (applying the same statistical approach than for BPA) were found between the analysed samples age and the amount of additives recorded. Obviously, the occurrence of additives depends on the plastic composition that is linked to the producer and the lot. According to migration results, it seems that older samples released less amount of additives. A possible explanation of this behavior is that such substances were not widely employed in past years, as confirmed by data about total content of additives measured after experiments providing the total PC dissolution (Bignardi et al., 2014). Besides, damage of surface was not found to be linked to additives release.

3.2. Non-intentionally added substances (NIAS)

Migration tests were carried out by employing two different food simulants, ethanol 95% (v/v) and isooctane. Since no standard corresponding to the identified molecules could be available, peak areas normalized respect to the surface in contact with simulant were used for a semi-quantitative approach.

Products of degradation

The presence of several oligomers derived from PC chain was evidenced in the examined simulants. The molecules identification was carried out by reconstructing the mass fragmentation spectrum of the most representative molecular ions visible in the full-scan chromatograms, as reported in a previous study (Bignardi et al., 2014).

Previous literature studies about polycarbonate ageing induced by light identified some of these molecules as PC-degradation products (Jang and Wilkie, 2004; Collin et al., 2012). The molecular weight values, comprised between 227 and 989 g/mol suggest that their ingestion may represent a possible risk for health.

From a qualitative point of view, a similar chromatographic profile was recorded for both simulants employed, although all signals found in isooctane were of lower intensity

A remarkable interesting point was that the pattern of degradation products released by old and new PC objects was different. A previous study (Bignardi et al., 2015) reported higher content of BPA being released from old samples, while there are no studies exploring a possible correlation between ageing and amount of other degradation products. In order to put in evidence possible differences, the values of peak areas detected in ethanol 95% were considered for all molecules, and evaluated in relation to the age and the degree of damage of each sample.

The peak area values of four of the molecules identified were correlated with sample age, surface damage degree and BPA. It could be seen that, obviously, high correlation can be observed between damage degree, age, and BPA amount, according to that described elsewhere (Bignardi et al., 2015). As for larger fragments, it could be seen that all peak areas considered were well correlated with age: in details, a high positive correlation was found with two molecules (481.2020 and 735.2963 m/z), and a sensible negative one with two other compounds (366.1700 and 486.1911 m/z). In particular, the first couple of molecules (481.2020 and 735.2963 m/z) was also correlated with damage degree and BPA amount. Therefore, the observed correlations allowed to group together two pairs of analytes showing similar features.

Identification and migration of colouring agents

Traces of colouring agents, not belonging to NIAS, but considered as unwanted migrants, were identified in migration experiments of some analysed samples, precisely in the red (solvent red 179), the orange (solvent Yellow 184) and the yellow ones (solvent Yellow 232). Molecules found were belonging to the category of solvent dyes. Blue and white samples did not release any dye since they were probably coloured by inorganic pigments.

Migration experiments were performed three consecutive times in both ethanol 95% and isoctane. Different peak intensity was obtained for the molecules in the two simulants. The amount of extracted dye was found to decrease during consecutive experiments in both solvents, but was still detectable until the third experiment only when ethanol 95% was employed. Therefore, ethanol was more effective in extracting the dye than isooctane.

To evaluate the eventual effect of the ageing on dye release, experiments on two samples of the same shape and colour (yellow) but different age (2013 vs. 2007) were performed. In Figure 2, the chromatograms showing the peak referred to the yellow dye (identified as solvent Yellow 232) found in three consecutive migration experiments performed in ethanol 95% are reported.

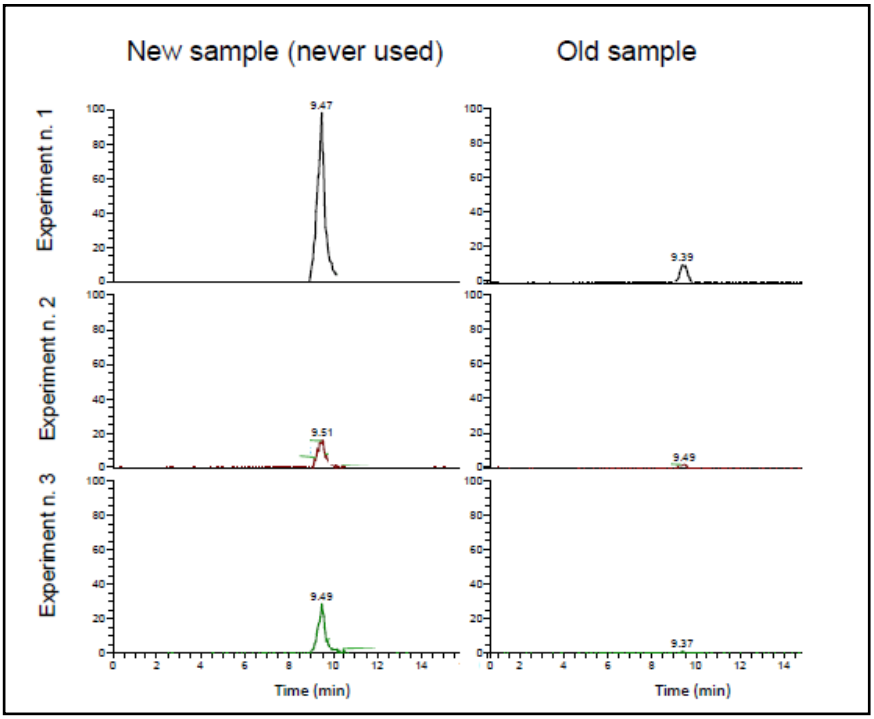

Figure 2. Peaks of the colouring agent Solvent Yellow 232 during three consecutive migration experiments in ethanol 95% .

Signals corresponding to the yellow dye are present in both samples, in all experiments, and were found to decrease from the first to the third migration. From the comparison between the two samples it is evident that the new sample releases higher amount of dye. A similar result was obtained by the analysis of two equal orange samples differing only for the age (2014 vs 2009). These data, although limited to a restricted number of samples, suggest that new plastic objects may act as potential source of dye release. It would be interesting to investigate in a deeper way

such behavior, since according to the national regulation of many countries organic dyes should not migrate into food simulants (Council of Europe, 1989).

3.3. Determination of polycarbonate additives, degradation products, and colorants in chocolate

Optimisation of the procedure for sample preparation

Dark chocolate used for the experiments contained 70 % of cocoa and represents a very complex matrix, containing 46 % of fats, 45 % of carbohydrates and 9 % proteins. Therefore, in order to extract the analytes of interest and reduce interferences, it was mandatory to set up a sample pretreatment.

Since no previous studies on procedure extraction of plastic additives from chocolate are reported in literature, the paper of Silva et al. (Silva et al, 2007), dealing with the migration of a model substance (diphenylbutadiene) from low-density polyethylene in foodstuffs with high fat contents (chocolate, chocolate spread and margarines) was considered as starting point.

Many additives can be used in plastic to enhance its characteristics, and they can be very different from each other in terms of physico-chemical properties. For this reason, the use of only one model substance to test the extraction procedure in terms of recovery would be not sufficient. The following molecules characterized by different degree of hydrophobicity, to be representative of the different classes of compounds possibly occurring, were selected between the most common additives found in plastic foodstuffs, on the basis of previous works (Bignardi et al, 2014; Bignardi et al, 2017): Cyasorb UV5411, Tinuvin 234, Uvitex OB, Tinuvin 327. BPA was also considered, as it is the monomer of polycarbonate and was found to migrate in many stimulants (Kubwabo et al., 2009).

A measured amount of each compound was added to the blank matrix in order to evaluate the recovery yield of the tested procedures.

Some of the analytes were moderately apolar, so their extraction from fatty matrices poses an important problem due to the co-extraction of the lipid components, mostly represented by triacylglycerols. Thus, the application of different clean-up procedures was required. Two techniques in particular are usually applied: the liquid–liquid partition with ACN and clean-up with solid phase extraction (SPE). The complete sample processing was characterized by the three following steps:

1. Dissolution of the chocolate with solvent: the extraction capacity of three different polarity solvents was evaluated: methanol, hexane, diethyl ether. The best solvent was found to be diethyl ether, allowing to obtain good extraction yield for all the selected standards, while very low recovery was obtained, especially for BPA, with the other solvents.

2. Liquid-liquid extraction: the extractive capacity of two solvents with different polarity, methanol and acetonitrile, was evaluated. The best solvent was found to be acetonitrile, since it allowed to obtain good extraction yield of the standards selected. Sonication for 1 minute was sufficient for exhaustive extraction.

3. Clean-up step to separate the interfering components of the matrix: different SPE cartridges (Florisil, C8 and C18) were tested. Several fractions of the eluate were collected and analysed to evaluate the optimal solvent composition to be used to for the elution of each analyte, and to evaluate the necessary amount of solvent.

The final method involved diethyl ether extraction followed by three steps of liquid-liquid extraction with ACN, and C18 SPE clean-up performed eluting with 10 mL of acetonitrile (two times), followed by resuspension of the extract with acetonitrile. Values of recovery were as follows: BPA 93±1.8 %, Tinuvin 234 90±8.2 %, Tinuvin 327 70±1.5 %, Cyasorb UV5411 78±1.1 % and Uvitex OB 88±1.2 %.

Another step was to verify if the extraction method was efficient also for the extraction of BPA-polycarbonate degradation products and the organic colorants identified in PC extract of previous work (Bignardi et al., 2014). Since no standard of these molecules were available, 1 g of chocolate was spiked with 100 µL of PC extract of four plastic samples containing a different colorant: yellow, orange, pink and red, and submitted to the optimized extraction procedure. The peak areas of the four main degradation products found during migration experiments, and of the colorants, were compared with those obtained after spiking the extract of a blank matrix. BPA-degradation products extraction yields was in the range 90±3-96±4%. Good extraction yields were registered also for organic colorants: 91±1-98±4%, except for solvent red 135 60±2%.

Optimisation of ESI and acquisition parameters by experimental design

The most important parameters affecting sensibility can be considered at two different levels: a first one regarding analyte ionization at source level, and a following one related to signal acquisition modality. The performed study considered both of them, trying to measure the effect

of the eventual combinations between the possible settings by means of a chemometric approach, that permits to take into account the potential interactions between the parameters to be adjusted.

A first study was aimed at exploring the most important parameters affecting ESI response. Analyte ionization at the electrospray interface level is known to be a very crucial step affecting sensitivity, and depends on many factors. In order to evaluate the influence of the main operating parameters of ESI source on the peak area of the six standards in matrix, the following four main factors were optimized by a chemometric approach: sheat gas (X_1, in the range 5-15 a.u.), sweep gas (X_2 in the range 0-2 a.u.), spray voltage (X_3 in the range 2.5-4 kV.) and S-lens voltage (X_4 in the range 20-80 V).

A two level full factorial design 2^k was carried out to determine the influence of these selected factors and their interactions.

It was found that the S-lens voltage is the parameters that affected the ESI response most severely. It had a positive effect upon peak area for all analytes. Increasing spray voltage also increased peak area of all analytes, except for BPA (maximum sensitivity reached by setting it at 2.5 kV). No interaction between sheat gas and spray voltage was found. The sheath gas was found not to affect ionization process in the case of Tinuvin 327 and BPA, and gave best results when set at a low level (5 a.u) for the other analytes. Similar results were obtained for sweep gas, for which the higher value was necessary only to enhance detection of Tinuvin 327.

Optimisation of tSIM-ddMSMS acquisition parameters by D-Optimal design

Several research groups evaluated MS settings during DDA mode in order to examine their significance in the experimental outcome and optimize instrument performance for a successful analysis. In terms of MS parameters the effect of dynamic exclusion (Guo et al., 2015; Li et al., 2016), signal thresholds setting to trigger an MS/MS event (Li et al., 2017), mass resolving power (Bignardi et al., 2017), ion injection time, number of MS/MS, monoisotopic precursor selection (Li et al., 2016), preview mode for FTMS scan (EURACHEM Guide, 1998), normalized collision energy (Li et al., 2016), Automatic Gain Control (AGC) target for MS and MS/MS scans, and number of microscans (EURACHEM Guide, 1998), have been evaluated and discussed, aiming to examine the significance and effect of instrument settings on identification rates. These reports revealed that MS settings influence identification rates and improved results can be obtained by optimizing instrument performance (Kalli et al., 2013). Those parameters have been studied separately, without taking into account potential synergies between them. However, it has to be considered that at least some of them could depend from each other and be affected by the other settings.

Basing on our experience, tSIMddMSMS acquisition is the best choice for quantitative purpose with quadrupole orbitrap, since permits to combine the excellent sensibility at tSIM level and at the same time the confirmation of the presence of the characteristic fragments of each analyte at MSMS level. This is crucial in complex matrix. For setting instrument method, the two most important parameters are AGC target and Resolution both for tSIM and ddMSMS, which are discrete variables with defined levels. AGC provides automated regulation to a dynamic ion flux transmitted from the source of the instrument (common in liquid chromatography (LC) coupled MS experiments), resulting in a more constant ion population in the mass analyzer (Page et al., 2005). The resolving power of the Orbitrap mass analyzer diminishes as the square root of the m/z ratio (Campo & Pico, 2015; EFSA Journal, 2016).

Acquiring data at higher resolving power results in better mass accuracy, but the scan duration increases with increasing resolving power. Therefore, the best compromise between speed, accuracy and identification rates has to be reached.

A chemometric approach has been exploited for the selection of the proper combination of the parameters giving the best response in term of peak area during a tSIMddMSMS acquisition.

Different types of experimental designs can be applied as factorial designs, appropriate for assessing main effects and interactions. In order to reduce invested resources (in terms of time, reagents and sample), the experiments to be performed were selected according to the D-optimality criterion. D-optimal designs have the property that the estimations of the coefficients of the model are the most precise possible.

The parameters Resolution SIM and Resolution MSMS, were chosen at two levels, low (35000 and 17500, respectively) and high (70000 and 35000, respectively), and the other two parameters, AGC target SIM and AGC target MSMS were chosen at three levels, low (2e5), central (1e6) and high (5e6). It should be emphasized that the number of experiments needed using a full factorial design is 36. However, using D-optimal design the number of experiments was reduced to twenty (performed in duplicate). The peak area of each analyte has been used as response.

The results obtained were elaborated by principal component analysis (PCA) in order to understand which experiments gave the best response in terms of peak area. As suggested by PCA, the parameters set in the experiments 19 and 20 gave the highest peak area for BPA, Tinuvin 234, Uvitex OB and for Cyasorb UV5411, but not for Tinuvin 327. These two experiments were made with high Resolution SIM (70000) and AGC target SIM, and high MSMS ($5e^6$ and $1e^6$). Since BPA was the analyte with the lowest limit of detection and quantification, and taking into account that Tinuvin 234 and Uvitex are two of plastic additives most used, the experiment number 20 was selected as a compromise for method validation.

Moreover, it permits to perform MSMS experiments at a resolution level of 35000. Besides, our data demonstrate that a very performing quantitative analysis in terms of sensitivity can be performed also at high resolution (70000) by setting simultaneously a very high AGC target sim value ($5e^6$).

Validation in matrix

The combination of tSIM-ddMSMS acquisition and the two-level optimization of the acquisition parameters allowed to obtain an increment of sensibility of several orders of magnitude for the standards taken into account respect to acquisition in FullMS-ddMSMS with inclusion list (see values of LOD and LOQ reported in Table 2).

Table 2. LOD and LOQ evaluated in matrix (comparison between Full-MS and tSIM)

Analyte	Retention time (min)	LOD FullMSddMSMS (ng g^{-1})	LOD tSIMddMSMS (ng g^{-1})	LOQ FullMSddMSMS (ng g^{-1})	LOQ tSIMddMSMS (ng g^{-1})
Bisphenol A	6.75	18	2.5	60	8.0
Tinuvin 327	14.64	80	0.75	267	2.5
Cyasorb UV5411	12.27	100	0.75	333	2.5
Tinuvin 234	13.47	5	0.1	16	0.35
Uvitex OB	14.04	5	0.2	16	0.70

In Table 3, the results of the validation procedure in matrix used for quantitative purposes are reported

Table 3. Validation results obtained in matrix (ng g^{-1})

Analyte	Linearity range	Calibration curve	r^2
Bisphenol A	10-500	y=15540±244x	0.997
Tinuvin 327	2.5-500	y=(173558±3157)x-(434361±146433)	0.997
Cyasorb UV5411	2.5-500	y=(671595±7453)x-(1257273±345685)	0.999
Tinuvin 234	0.5-100	y=3941858±13202x	0.999
Uvitex OB	1-100	y=(6747784±37666)x+(3838984±10264)	0.999

The method accuracy was also tested in terms of precision (retention times and peak areas) and trueness. Very good precision in terms of intra-day repeteability was calculated providing RSD% values lower than 2 % both for area and retention times, intermediate precision results were found not exceeding 7 %, confirming good method precision. Matrix effect was investigate by comparison of the two slopes obtained from calibration curve built in solvent and that one built

by spiking standard mixture solution in blank matrix extract. The results evidenced a signal suppression/enhancement ranging from -60 % to +20 %.

Matrix-matched calibration curves, with fortification of chocolate before extraction, were built up to perform a label-free quantification method.

Sample analysis: migration experiments with chocolate

The applicability of the validated method was verified by analyzing chocolate put in contact with fourteen samples of tableware of different age and color. The same samples were analyzed in previous works using isooctane and 95 % ethanol as simulants, by performing three consecutive migration tests. In those experiments, the presence of the additives Cyasorb UV5411, Tinuvin 234, Uvitex OB, Tinuvin 327, and the monomer BPA was found in many samples (Bignardi et al., 2017; Bignardi et al., 2015) . Results recorded in chocolate are reported in Table 4, and show traces of plastic additives in experiments involving only the Red 2012 and Orange 2009 samples, which were characterized by the highest amounts of plastic additives during migration tests with simulants. Traces of BPA were found only in samples Orange 2009 and Transparent 2000, the samples releasing the higher levels of BPA in simulants. Regarding organic colorants, only the organic colorant named Solvent yellow 184 was detected after migration from the Orange 2014 (new sample, never used previously). Quantitative analysis showed reduced amounts of the substances migrating to chocolate in comparison to simulants, probably because of the higher density of melted chocolate respect to the liquid simulants.

Table 4. Results of analyzed samples (ng/g of chocolate)

Sample name	BPA	Tinuvin 234	Tinuvin 327	Uvitex OB	Cyasorb 5411	Colorants
White 2013	-	-	-	-	-	-
Yellow 2013	-	-	-	-	-	-
Blue 2013	-	-	-	-	-	-
Green 2012	-	-	-	-	-	-
Red 2012	-	0.339±0.027	-	0.060±0.002	-	-
Dark 2011	-	-	-	-	-	-
Blue 2010	-	-	-	-	-	-
Orange 2009	<LOQ	0.614±0.032	-	-	0.399±0.018	-
Orange 2014	-	-	-	-	-	*Solvent yellow 184 (traces)*
Yellow 2007	-	-	-	-	-	-
Transparent 2006	-	-	-	-	-	-
Transparent 2000	<LOQ	-	-	-	-	-
Transparent 1996	<LOQ	-	-	-	-	-
Transparent 1999	-	-	-	-	-	-

An important finding regards the detection, for the first time, of BPA-PC degradation products in chocolate samples. The detected molecules, whose presence was already found in previous experiments with stimulants (Bignardi et al., 2017) had the following m/z: 486.1911, 481.2020, 735.2963, 366.1700. Their identity corresponded to oligomers of polycarbonate chain and was established through a search processed by software elaboration starting from the accurate mass recorded. The comparison of isotopic patterns provided by a simulator allowed to achieve the unequivocal identity between the potential formulas.

4. CONCLUSIONS

The validated analytical method has been successfully applied to the analysis of food liquid simulants put in contact with the samples objects of the study, and shown to be suitable for the identification and quantitative determination of the compounds of interest. Data analysis demonstrated a significant correlation between aging and BPA release, higher than that recorded between BPA and surface damage. These data show that ageing, linked to light exposure, is more effective for polymer degradation than usage. Therefore, the employment of too old food containers should be discouraged, although the levels of BPA found to migrate were in all cases below the limits imposed by regulation. Similarly, migrating amounts of the additives were always below the allowed limits. No correlation with age and surface damage was recorded.

This work shows the high potential of the technique employed and open new perspectives to evaluate the possible migration of PC degradation products constituted by dimers or oligomers.

Regarding the Study on plastic re-usable containers aimed at storing food are very limited and should be encouraged since normally the tests for checking material suitability are only performed on new samples, and are not repeated after ageing. Since these objects are usually employed during many years, despite ageing and surface damage, they obviously undergo to degradation, and may become a potential source of NIAS. The molecules generated by material decomposition can act as possible contaminants as they can easily reach the food products. It is the first time that PC degradation products have been identified in food simulants; besides, a peculiar behavior has been observed for different groups of compounds, suggesting the presence of two groups of oligomers diversely correlated with age and surface damage.

It is also the first time that colouring agents have been found to migrate in simulants, and this behavior should be deeply investigated since they may represent a possible source of food contaminants.

It can be concluded that the high potential of High Resolution Mass Spectrometry has been shown, in particular for its capability to identify untargeted compounds. It also opens new perspectives suggesting the possibility to enhance its sensitivity by optimizing parameter settings by chemometric approach. The optimized method was characterized by improved features in terms of sensitivity respect to existing methods

Advance

The discovery of the presence of substances of emerging concerns such as polycarbonate degradation products and unreacted oligomers in chocolate after contact with tableware represents a challenge in the field of research of molecules representing substances of emerging concern and non-intentionally added substances (NIAS).

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COLLATERAL ACTIVITY

In parallel to the research activity described in the previous chapters, a collateral activity, based on the use of an Oxitest reactor to evaluate the oxidative stability of food products, has been carried out.

The level of lipid oxidation can be measured by direct methods (chemical or physical) that are often time consuming, expensive, and require trained people. Indirect methods are an alternative to evaluate the oxidation degree of fatty foods through tests, which measure the fatty food's stability under accelerated conditions as elevated temperature. In this way, the obtained autoxidation measurements are performed in a few hours instead of weeks or months. Normally, these accelerated tests provide an oxidation curve, characterized by an Induction Period (IP), which measures the time required to reach an end-point of oxidation that corresponds either to a level of detectable rancidity or a sudden change in the rate of oxidation.

The oxidative stability of different samples has been investigated by accelerating the oxidation process through the use of Oxitest reactor (Velp Scientifica), an instrument that speeds up the oxidation process based on two accelerating factors: temperature and oxygen pressure. Compared to similar and conventional technologies, the Oxitest reactor is easy to use. Its big advantage is that it allows analysis directly on the food (liquid, solid, or pasty) without previous fat separation from the matrix.

The instrument measures the absolute pressure change inside two thermostated titanium chambers and monitors the oxygen uptake by reactive components in the samples. At the end of the analysis, an Induction Period (IP) value is automatically generates for each chamber. The higher IP values correspond to longer oxidative stability of the product tested over time.

The following food products have been investigated using Oxitest reactor:

- ✓ **Olive oils enriched with natural extract**

The increase of oxidative stability of olive and extra-virgin olive oils enriched with essential oils (oregano and rosemary) and red chilli pepper powders (sweet and hot), has been investigated. These natural extract have been added in small amount (1 % w/w for spices and 3 % w/w, respectively), on the basis of preliminary experiments. Measurements were performed by evaluating stability of blank oils (without any additions) compared to enriched oils.

- ✓ **Olive oils stored in different packaging materials**

The performance of two polypropilene based materials with oxygen barrier compared with those of a glass container have been evaluated.

- ✓ **Tuna fish fillet in olive oil**

Tuna fish samples packaged in glass containers, and stored for different time have been analysed.

- ✓ **Biscuits prepared with different flour mixtures**

A study on biscuits prepared in the Department of Food and Drug of the University of Parma (Dr. Massimiliano Rinaldi), and based on the use of gluten free mixtures additioned to chestnut flour, was carried out.

Main findings

Shelf-life of olive oil and extra-virgin olive oil can be increased by adding natural extracts (such as essential oil end sweet red pepper powder) in small amount, without affecting the organoleptic characteristics of the final product.

Olive oil stored in glass showed the higher stability against oxidability (higher IP value). One of the polypropilene material showed a better resistance to permeability since results on olive oil stored in this container presented a less marked variation in IP value. Duration time of thermal treatment influenced the IP value of the product: IP value was higher when treatment was milder.

Regarding results on tuna fish, a progressive increase of product oxidative stability with time, accompanied by a parallel decrease of the olive oil stability it has been shown. It is possible that the antioxidant compounds occurring in olive oil exert a protective action on the soaked tuna fish fillets.

An interesting conclusion is that tuna fillet shelf-life is prolonged after its storage in olive oil, and even increases its quality, in terms of oxidative stability, with time.

About the measures performed on biscuits, it can be seen that the flour mixtures containing chestnut gave higher IP values than controls, since chestnut flour is rich of antioxidant compounds. Obtained data showed good and promising results since percentages of chestnut flour in the dough lead also to a product with improved colour, harder texture and better taste, opening new perspectives in the use of chestnut flour for preparation of gluten-free products.

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