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Functional Food Development through Novel Sustainable Processing and Formulation

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“Si studia, si studia, si studia ancora, poi un giorno, studiando, c’è una strana sensazione:
ma no, non può essere così, qui c’è qualcosa che non torna.
Da quel momento, sei uno scienziato.”

Carlo Rovelli - Ci sono luoghi al mondo dove più che le regole è importante la gentilezza

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Preface

During the last decades, food researchers have focused their efforts to study foods not only in terms of safety and quality but also to understand their potential health benefits. The health promoting capacity of food has been known for long time and can be effectively summarized by the famous Hippocrates' claim "let food be thy medicine and medicine be thy food".

Food intake entails the ingestion of a wide variety of components that beyond basic nutrition may providing multifaceted functions towards health protection. On the contrary, even if artificial drugs are precisely designed to nurse specific diseases, they often carry undesired side effects, thus impinging their suitability for the whole population. Additionally, consumers are nowadays raising their awareness towards healthy diets, able in some cases to reduce or avoid drug intake.

Indeed, research has already provided promising results about the efficacy of some foods in tackling several chronic diseases. However, these results have been largely collected by analysing model systems, whereas little is known about their functionality in real food systems.

This Ph.D. thesis aimed at assessing the effect of conventional and unconventional technological interventions on the functionality of some popular food products, to identify the most effective interventions (processing and formulation) able to maintain and possibly enhance the effectiveness of bioactive compounds in foods, yet guaranteeing safety and sensory requirements.

Summary

The interest in functional food has experienced a steep increase in the last years. However, this is not recognized as an independent category and no dedicated regulation exists. This gap jeopardizes the effective operability of the functional food development cycle.

Food design is one of the major steps in building this cycle and relies on the activity of food technologists. However, to evaluate the efficacy of applied interventions the competences of other actors, such as nutritionists, are required. Still, matching these two technical aspects is missing. Thus, in this Ph.D. thesis, an approach to drive functionality based on the integration between the technological and the nutritional viewpoint is proposed. In particular, different technological interventions, based on conventional and unconventional processing and formulation were applied to different food matrices, known for their natural antidiabetic potential due to the content in bioactive compounds, such as turmeric, coffee and apple derivatives. The antidiabetic capacity was assessed through *in vitro* tests, followed by cell cultures and finally animal models. Moreover, the combination of different food and between food and drugs was tested to identify potential synergies.

Acquired results showed how food functionality is dramatically affected by food matrix, by the technological interventions, intended as formulation and processing, as well as by co-ingested components and by the digestion process. The efficacy of some technologies in bolstering the antidiabetic capacity of foods, as well as the possibility to potentially reduce drug dosage by proper combinations with functional foods, was demonstrated. Still, the molecules and the mechanisms underlying this functionality are partially unrevealed. This knowledge would provide a tool to drive functionality by targeted interventions, based on a holistic approach. The latter requires teamwork encompassing all the actors involved in the functional food development cycle.

Chapter 1 Introduction

1.1 Healthy and sustainable food system: background overview

During the last years, the need to redefine the boundaries of food production has come to light. We are currently dealing with major and opposite issues. On one hand the exponential increase in worldwide population is posing at risk the food security especially in low income and developing countries (Godfray *et al.*, 2010). On the other hand, we are facing a dramatic increase in chronic diseases, caused by excessive food intake or an unbalanced diet (WHO, 2014).

To handle these trends, it is necessary to look in a more integrated way at the current food system (de Vries, Axelos, Sarni-Manchado, & O'Donohue, 2018). This is defined as a framework considering all the elements (i.e. environment, people, inputs, outputs, processes, infrastructures, institutions) and activities related to primary production, processing, distribution, preparation and consumption of food, also taking into account the socio-economic and environmental outcomes of these activities (Perrot *et al.*, 2016).

To prevent food insecurity, it is essential to establish some limits such as avoiding the unnecessary exploitation of resources, which is possible by applying different strategies, such as: consuming low density-high satiating food and using alternative protein sources; efficiently transforming and using resources, that is feasible through targeted and eco-efficient processes; valorizing processing by-products, by applying the food waste pyramid (Manzocco, Alongi, Sillani, & Nicoli, 2016; Plazzotta, Manzocco, & Nicoli, 2017).

Thus, designing healthy products requires food product engineering to operate within environmental and health-related boundaries, in order to deliver on the market healthy and at the same time sustainable foods (Willett *et al.*, 2019). To this purpose, research is focusing on the application of conventional and unconventional processes and on the exploitation of

processing by-products as bioactive ingredients to reduce the impact of food production while enhancing functionality (Van Der Goot *et al.*, 2016).

1.2 Functional food: historical overview

The term "functional food" appeared for the first time in Japan in 1984 (Martirosyan & Singh, 2015). The Japanese government defined a new product category, Foods for Specific Health Uses (FOSHU), producing a dedicated legislative framework, the so-called Nutrition Improvement Law (Ministry of Health, Labour, and Welfare - MHLW, 1991).

FOSHU are defined as "foods containing an ingredient with functions for health and officially approved to claim its physiological effects on the human body. FOSHU are intended to be consumed for the maintenance/promotion of health or special health uses by people who wish to control health conditions, including blood pressure or blood cholesterol. In order to sell food as FOSHU, the assessment for the safety of the food and effectiveness of the functions for health is required, and the claim must be approved by the MHLW".

Japan was followed by the United States that in the '90s developed the first act relevant to health claims (Greenberg, 1990), involving the technical support of FDA (Moors, 2012), but did not provide a formal definition of functional food (Martirosyan & Singh, 2015).

European countries acquired the concept of functional food more than 10 years later, when the European Parliament and Council introduced the regulation on nutritional and health claims (Reg. (EU) n. 1924/2006), but also in this case without mentioning a formal definition for functional foods.

The research interest towards functional foods thus experienced a steep increase only in the 21st century, recording an exponential increase (from 500 to 5000/year) in published papers between 2000 and 2018 (Figure 1).

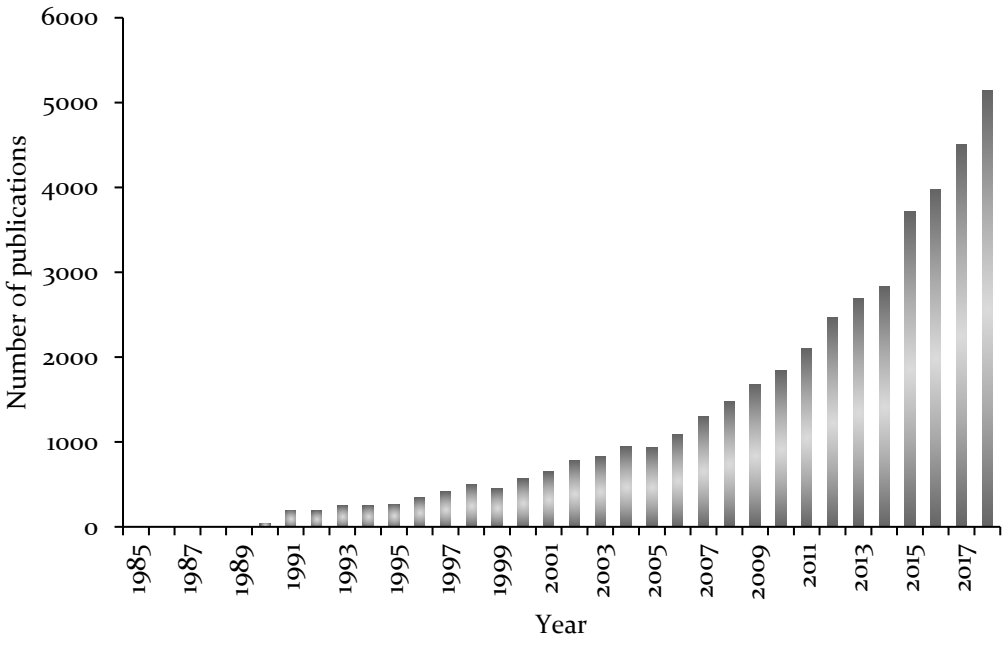


Figure 1. Number of papers published between 1985 and 2018 containing the keyword "functional food".

This globally growing interest and acceptance of functional foods has tremendously influenced their market (Kaur & Das, 2011). In fact, the global market of functional foods presents an annual growth rate of almost 10% and is estimated to exceed $300 billion by 2020 (Research and Markets, 2015). Still, many stakeholders, among which are researchers, in the European Union are confused about this topic, due to the lack of a definition and a dedicated regulation, and this, in turn, jeopardizes the effective development of functional foods.

1.3 The "Functional food development cycle"

When dealing with functional food development, several steps need to be considered to manufacture a successful product (Gur, Mawuntu, & Martirosyan, 2018). These steps are interconnected and can be regarded as part of a "functional food development cycle" (Figure 2).

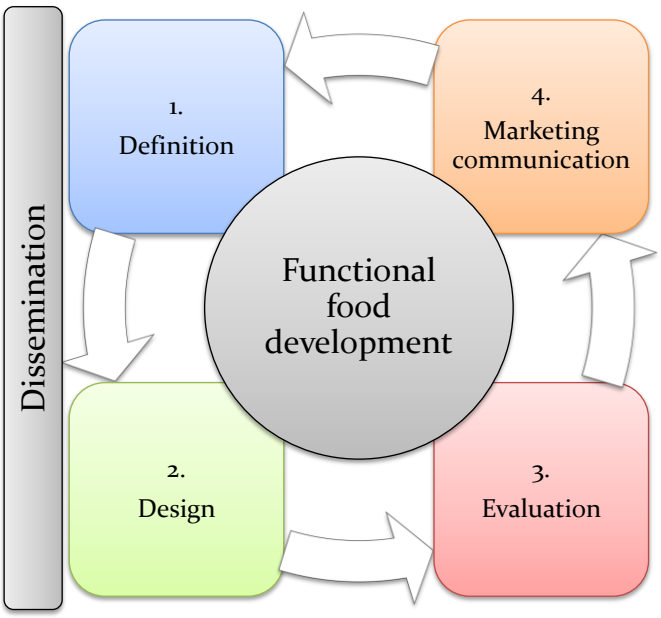


Figure 2. Functional food development cycle.

As reported in Figure 2, the phases building up the functional food development cycle are sequential. The preliminary step, essential to guide functional food design, is its definition. Once the target is known, it is possible to manage the following steps. The design represents the technological core of functional food development. The efficacy of technological interventions needs then to be assessed through the evaluation step. Later, a proper marketing communication strategy is required to effectively deliver the product on the market. The knowledge acquired along the chain allows to further detail functional food definition. Indeed, all these steps are interconnected by the dissemination activity, which is essential to guarantee an effective cycle functioning.

1.3.1 Definition

Although the research interest in the functional food field is exponentially growing, the lack of a comprehensive picture on this topic is critical and has produced a debate on the definition of functional food that is still ongoing.

The American Diabetes Association (ADA) classifies all foods as functional at some physiological level since they provide nutrient, sustain growth, or maintain vital processes (American Dietetic Association, 2009). However, the term "functional food" generally refers to food products providing a specific beneficial effect towards health, going beyond basic nutrition. From this perspective, as highlighted by several authors (Aronson, 2017; Gur *et*

al., 2018; Santini *et al.*, 2018), many definitions have been applied within the field of foods providing beneficial effects towards health (Table 1).

Table 1. Terms and definitions relevant to the functional food field.

Term	Definition	Reference
Food supplement	Food product whose purpose is to supplement the normal diet, and which consists of a concentrated source of nutrients or other substances with nutritional or physiological effects, single or in combination, marketed in dosed formulations, such as capsules, tablets, or pills, designed to be taken in small individual measured quantities	Dir. 2002/46/EC
	A product in the form of a capsule, powder, soft gel or gel cap intended to supplement the diet to enhance health that bears or contains one or more of the following dietary ingredients: a vitamin, mineral, amino acid, or other botanical or dietary substance	Zeisel, 1999
Dietary supplement	A substance added to the diet, often taken as a pharmaceutical formulation, to treat or prevent a deficiency, or to enhance growth, health, or well-being	Aronson, 2017
Fortified food	Foodstuffs to which compounds of proven therapeutic or preventive efficacy have been added	Aronson, 2017
Food for medical use	Complete nutritional food with a formulation of nutrients standards, which may constitute the sole source of nutrition for the person to whom it is addressed. Or alternatively: complete nutritional food with a formulation of nutrient adapted to a specific disease, disorder or medical condition, which may constitute the only source of nutrition for the person to whom it is addressed. Or alternatively: nutritionally incomplete food with a formulation of standard nutrients or adapted for a specific disease, disorder or medical condition, which it is not suitable to be used as the only source of nutrition	Dir. 1999/21/EC
Nutritional medicinal product	Foods or formulations containing nutritional ingredients from which specific nutrients have been omitted	Aronson, 2017
Phytochemical	Substances found in edible fruit and vegetables that can be ingested daily (in quantities of grams) by humans and that exhibit a potential to favorably modulate human metabolism to prevent cancer and other diseases.	Bloch & Thomson, 1995
Bioactive compound	Naturally occurring chemical compounds present in, or derived from a plant, animal, or marine source, that exert the desired health/ wellness benefit	Hardy, 2000
Functional ingredient	Standardized and characterized preparations, fractions or extracts containing bioactive compounds of varying purity, that are used as ingredients by manufacturers in the food	Hardy, 2000

Table 1. (continues)

Term	Definition	Reference
Nutraceutical	Food or part of food that provides medical or health benefits, including the prevention and/or treatment of a disease	De Felice, 1995
	A diet supplement that delivers a concentrated form of a biologically active component of food in a nonfood matrix to enhance health	Zeisel, 1999
	Any substance that is a food or a part of a food and is able to induce medical and health benefits, including the prevention and treatment of disease	Brower, 1998
	A foodstuff (as a fortified food or dietary supplement) that provides health benefits in addition to its basic nutritional	Merriam-Webster Online Dictionary, 2015
	Nutritional products that provide health and medical benefits, including the prevention and treatment of disease	Santini et al., 2018
Functional food	Foods that encompass potentially healthful products, including any modified foods or food ingredients that may provide a health benefit beyond the nutrients it contains	Thomas & Earl, 1994
	Foods that may provide a health benefit beyond basic nutrition	Day, Seymour, Pitts, Konczak, & Lundin, 2009
	Nutrient consumed as part of a normal diet but delivering one or more active ingredients (that have physiological effects and may enhance health) within the food matrix	Zeisel, 1999
	Product which is shown in a satisfactory manner that, in addition to adequate nutritional effects, induces beneficial effects on one or more target functions of the organism, significantly improving the health status and welfare or reducing the risk of disease	Diplock et al., 1999
	Any food or ingredient that has a positive impact on an individual's health, physical performance, or state of mind, in addition to its nutritive value	Hardy, 2000
	Foods and food components that provide a health benefit beyond basic nutrition. These substances provide essential nutrients often beyond quantities necessary for normal maintenance, growth, and development, and/or other biologically active components that impact health benefits	F. Clydesdale, 2004
	Natural or processed foods that contain known or unknown biologically-active compounds; which, in defined, effective, and non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic diseases	Martirosyan & Prasad, 2009
	Natural or processed foods that contain biologically active compounds; which, in defined, effective, and non-toxic amounts, provide a clinically proven and documented health benefit utilizing specific biomarkers for the prevention, management, or treatment of chronic disease or its symptoms	Gur et al., 2018

Based on Table 1, it comes to light that, although different terms in this field have been properly and univocally defined by EU and extra-EU legislation as in the case of food supplement, still no common and exhaustive definition has been recognized for functional food. The discussion on "functional food" definition has been ongoing for more than 20 years and is still opened. Actually, the terms nutraceutical and functional foods are often confused and interchanged (da Costa, 2017).

Different attempts have been made to propose a commonly agreed definition. To this purpose, the International Life Science Institute (ILSI), promoted a European Commission Concerted Action on Functional Food Science in Europe (FUFOSE) to propose a working definition of functional food, with the final aim of establishing a scientific approach based on evidences for their development (Diplock *et al.*, 1999) Similarly, the Functional Food Center (FFC) has been organizing a series of conferences dealing with the issue since 2004. The last proposal was the definition reported in Table 1 and presented by FFC in 2018 (Gur *et al.*, 2018). The main features of this definition are:

- ✓ the type of food ("natural or processed");
- ✓ the components from this food affecting physiological functions ("containing biologically active compounds");
- ✓ the quantity, efficacy, and level of toxicity that this food possesses ("defined, effective, and non-toxic amounts");
- ✓ the concrete evidence of the overall impact of its effects ("provide a clinically proven and documented health benefit");
- ✓ the identification of alterations in the body and signal of disease ("utilizing specific biomarkers");
- ✓ the three key elements which functional food tries to achieve: prevent, manage, or treat not only the disease itself but also its symptoms ("prevention, management, or treatment of chronic disease or its symptoms").

It can be noticed that, as compared to the previous FFC definition (Martirosyan & Prasad, 2009), major changes refer to the need to identify the bioactive compounds and specify which is the measured outcome to assess functionality.

As stated in this definition, both the technological ("natural or processed food") and the nutritional (functionality assessment) viewpoints are brought up.

Besides their definition, functional foods can be classified in different classes depending on their origin and eventual modifications (Kaur & Das, 2011):

- ✓ products fortified with ingredients having a positive influence on health;

- ✓ products cleared from anti-nutritional compounds;
- ✓ raw materials improved/fortified/cleared by changing feeding practices (animals) or postharvest treatments (fruits and vegetables);
- ✓ novel foods with an improved health benefit.

Based on these considerations, a natural traditional product containing components positively affecting health should not be regarded as a functional food (Kaur & Das, 2011). However, the most recently proposed definition includes in the category also this class of products (Gur *et al.*, 2018). By contrast, it should be noticed that this definition does not include those products cleared from some components (i.e. light foods), although these are generally intended as foods carrying a health-promoting effect.

Due to the confusion and misunderstanding occurring when dealing with functional foods, an urgent need is to advocate the experts to establish a standard functional food definition. This could substantially contribute to pushing the approval of a unique and agreed definition by governments (Gur *et al.*, 2018).

1.3.1.1 Current regulations applying to functional foods in Europe

Due to the lack of legitimation by the legislator, the term "functional food" is currently used mostly as a marketing idiom for the category (Martirosyan & Singh, 2015). Since no dedicated legislative framework has been developed in Europe for functional food yet, several regulations relevant to the food field apply to this category (Coppens, Fernandes Da Silva, & Pettman, 2006):

- ✓ Dir. 89/398/EEC, relevant to foods for particular nutritional uses;
- ✓ Reg. (EU) n. 178/2002, defining food and foodstuff and laying down the concept of food safety;
- ✓ Dir. 2002/46/EC, identifying food supplements;
- ✓ Dir. 2004/27/EC, relevant to medicinal products;
- ✓ Reg. (EU) n. 1924/2006, regulating nutritional and health claims;
- ✓ Reg. (EU) n. 353/2008, establishing rules for applications for authorization of health claims;
- ✓ Reg. (EU) n. 1169/2011, laying down labeling rules;
- ✓ Reg. (EU) n. 2015/2283, concerning novel foods.

However, a dedicated regulation establishing functional food as a separate category is urgently needed (Gur *et al.*, 2018), as the innovation system can only work efficiently within a well-defined framework. Ultimately, besides guaranteeing consumer protection, a

dedicated regulation can guide and control the innovation processes in the functional food field in terms of setting rules or standards for products and processes (Moors, 2012). Clearly elucidated rules could, in fact, result in an increase in the investments on functional food R&D due to the reduced fear of working in a grey area, producing an increase in the availability of new targeted functional products on the market (Kaur & Das, 2011).

A key aspect to be considered when dealing with functional food legislative framework is the need to clearly lay down the rules for the efficacy assessment. This issue has already been brought about by the Reg. (EU) n. 1924/2006 concerning nutrition and health claims and more in detail by the Reg. (EU) n. 353/2008 that establishes the requirements to be fulfilled in order to obtain the authorization for health claims.

Besides demonstrating the efficacy of the food for which the claim is applied for, another key feature to guarantee the approval of the claim is its intelligibility by the average consumer. This brings about the important need for effective dissemination (Figure 2). Only by educating the general public towards functional foods it is possible to make the consumer correctly understanding the information behind nutrition and health claims.

The scientific requirements underlying the approval of health claims cover a broad range of effects, including the immune system protection, the prevention of oxidative damage and cardiovascular diseases, the control of blood glucose levels, the health of bones and skin, the protection of nervous system and an overall improvement of physical performances (Reg. (EU) n. 1924/2006). Demonstrating such effects could result quite challenging and thus many reasons can lie beyond a rejection by EFSA. In particular, this occurs when (Reg. (EU) n. 353/2008):

- ✓ there is a lack of systematic literature review;
- ✓ criticisms in the study design have been identified;
- ✓ the claimed effect refers to a medicinal product more than a food with health properties (Dir. 2004/27/EC);
- ✓ the food/food constituent is not sufficiently characterized;
- ✓ the effects of the food matrix, processing and storage are not sufficiently characterized;
- ✓ the bioaccessibility, bioavailability, and bioactivity are not sufficiently characterized;
- ✓ evidence of the cause-effect relationship between the food/food constituent and the claimed effect has not been established.

Based on these considerations, some major weaknesses behind the development of functional foods come to light. Considering the functional food development cycle (Figure 2), the most critical steps are represented by design and evaluation. In fact, making clear the

cause-effect relationship is actually hindered by the complexity of food matrix which may disguise the observed effects, leading to misinterpret the outcomes of experimental trials.

1.3.2 Design

Although many proposals have been made for a common approach to deliver effective functional foods, based on a rational design from the molecular to the macroscopic level (McClements, 2009), the lack of a defined target hinders their implementation (Gur *et al.*, 2018).

The first step to develop a functional food is to identify a goal. This can be selected based on two opposite paths: one can be regarded as a top-down approach, deriving from a new idea to face food-related health issues. Although the innovative potential, this approach does not result in an adequate involvement of all the actors and might not fulfill consumer expectations. Thus, more favorably a bottom-up approach should be applied, in order to deliver effective solutions to food-related health issues, while guaranteeing consumer acceptability (Bigliardi & Galati, 2013; Bleiel, 2010; Gur *et al.*, 2018; Jeffery, 2005; Jones & Jew, 2007; Siró, Kápolna, Kápolna, & Lugasi, 2008).

In industrialized countries, because of rising medical costs and increased life span, nutritional recommendations formulated by health professionals have motivated the food industry to provide products that help consumers to be in line with these recommendations (Kaur & Das, 2011).

In the light of the most incident health-related chronic diseases, such as obesity, cardiovascular diseases, and diabetes, two are the major general targets to be achieved through food design (O'Sullivan, Barbut, & Marangoni, 2016). Table 2 reports these targets together with the strategies to be followed and the subsequent possible tactics to be implemented.

Table 2. Target, strategy, and tactic to be implemented to design functional foods.

Target	Strategy	Tactic
Reducing caloric intake	➢ decreasing macronutrient intake ➢ slowing down macronutrient digestion ➢ increasing the satiety feeling	➢ using naturally low caloric foods ➢ reducing conventional food portions ➢ formulating low caloric foods ➢ designing food structures able to slow down the digestion rate
Introducing foods with healthy properties	➢ enhancing micronutrients absorption	➢ using foods naturally providing bioactive compounds ➢ formulating foods enriched with bioactive compounds

As reported in Table 2, a reduction in the energy intake can be achieved by decreasing the intake of macronutrients or by slowing down their digestion, thus maintaining their concentration constant in the bloodstream. This, in turn, would increase the satiety feeling, playing an indirect effect on caloric intake (Ruijschop, Burseg, Lambers, & Overduin, 2009). The tactics to be followed to reach this target may be different and include simple dietetic approaches, such as the use of naturally low caloric foods and the control of conventional food portions, but most often a technological intervention is required, specifically through the formulation of low caloric foods or the design of structures able to slow down the digestion rate (Q. Guo, Ye, Bellissimo, Singh, & Rousseau, 2017).

The other major target, namely consuming foods with healthy properties, results in the delivery of micronutrients, such as phenolic compounds, which may play an active role towards the prevention and management of diet-related chronic diseases (Williamson, 2017). The target can be gathered through a simple dietetic intervention, i.e. ingesting foods naturally providing bioactive compounds, or may be pursued by the formulation of foods enriched with bioactive compounds.

Following the general targets to be achieved through food design (Table 2), the functional foods obtained upon a technological intervention can be classified into two main categories:

a. **light food**, in which the concentration of an undesired component is reduced (e.g. low fat, low sugar);
b. **bioactive-rich food**, namely a product in which the delivery of bioactive compounds has been bolstered (e.g. "high protein", "source of fiber", "enriched vitamins A, D, B_{12} and calcium").

It should always be kept in mind that to claim the "light" or "enriched" state of food, its properties must fall within the requirements reported in Reg. (EU) n. 1924/2006. Any new claim must be approved following the Reg. (EU) n. 353/2008, before being used.

Food engineering has proposed a wide variety of approaches to modify the digestion rate of macronutrients and to enhance the delivery of micronutrients and bioactive compounds.

To obtain light foods, the primary technological approaches are:

- ✓ **increasing the non-caloric fraction**. This mostly refers to the entire, or most commonly partial, replacement of triglycerides and carbohydrates with water and air (McClements *et al.*, 2015). This approach should take into account changes in the physical and chemical properties of the product, affecting its stability and sensory properties. Thus, the food structure must be modified accordingly to mimic the rheological properties of conventional food;

- ✓ **removing undesired components** (alcohol, caffeine, cholesterol) when this is compatible with the food stability and acceptability. When the removal of an undesired ingredient is responsible for a decrease in the food stability and acceptability, due to microbial growth, physical and sensory changes, as in the case of sucrose, lipids, gluten, sodium, lactose, it is necessary **replacing** them with compounds exhibiting sensory and functional properties analogous to those of the component they are intended to substitute (Giarnetti, Paradiso, Caponio, Summo, & Pasqualone, 2015; Zoulias, Oreopoulou, & Tzia, 2002). It should be kept in mind that when the formulation is modified, a one by one substitution is difficult to be achieved and thus a combination of different ingredients is often preferred. This requires also the processing condition to be redefined.

On the other hand, the technological approaches to obtain bioactive-rich foods are:

- ✓ **bolstering the delivery** of bioactive compounds naturally present in the food. This is possible through the induction of binding interactions with other compounds present in the food matrix, as well as by changing the physical state of the food matrix to let it play a protective role towards bioactive compounds (Z. Zhang, Zhang, Chen, Tong, & McClements, 2015);
- ✓ **enriching** the food with bioactive compounds (as extracts or as part of a matrix). Bioactive rich extracts are often encapsulated, embedded, or delivered through coating and clustering (McClements & Li, 2010);
- ✓ **inducing the formation** of bioactive compounds upon processing (Saeeduddin *et al.*, 2016).

Despite the wide variety of strategies available to steer the functionality of food ingredients, a major hindrance in the implementation of newly developed products at the industrial level, i.e. their application in a real food product, lies in the lack of knowledge relevant to the complexity of food matrix (Parada & Aguilera, 2007). In fact, most interventions do not take into account the interactions among food components occurring within the matrix upon formulation. This might result in the development of a functional ingredient that is effective when assessed alone but behaves in an unpredictable manner when used within a food formulation due to antagonist, additive or synergistic effects among components (Messina *et al.*, 2001).

However, the interaction among different components occurring within a complex food matrix should not be regarded as negative. In some cases, synergies can occur, leading to an enhancement in the bioactive effect (Jacobs, Tapsell, & Temple, 2011). This can be considered

as a further tool available for food engineering to steer functionality by taking advantage of the complexity of food matrix (Parada & Aguilera, 2007). Researchers are actually becoming aware of this option. In particular, this approach is referred to as the use of excipient foods/ingredients, namely foods that may have no bioactivity themselves but boost the efficacy of a bioactive agent (McClements *et al.*, 2015).

To this regard, another crucial aspect to be considered is the co-ingestion of functional foods with other foods within a meal and/or with the drugs used to treat chronic diseases (Melchior, Alongi, Boschelle, & Anese, 2019). The combination of food products among them or with drugs further increases the complexity of the system under investigation, as additional interactions may occur. Only few studies currently deal with this aspect, and most of them still investigate model systems (e.g. bioactive compound extracts), especially when dealing with drug combination (Boath, Stewart, & McDougall, 2012; Gao, Xu, Wang, Wang, & Hochstetter, 2013).

In addition, together with food matrix interactions, another key feature affecting the functionality is processing. Although it is well known to substantially affect food composition and the interactions occurring among components, both from a quantitative and a qualitative viewpoint, few studies deal with the effect of conventional, and even more so unconventional, processing on food functionality, as they mostly focus on the formulation step (Figure 3).

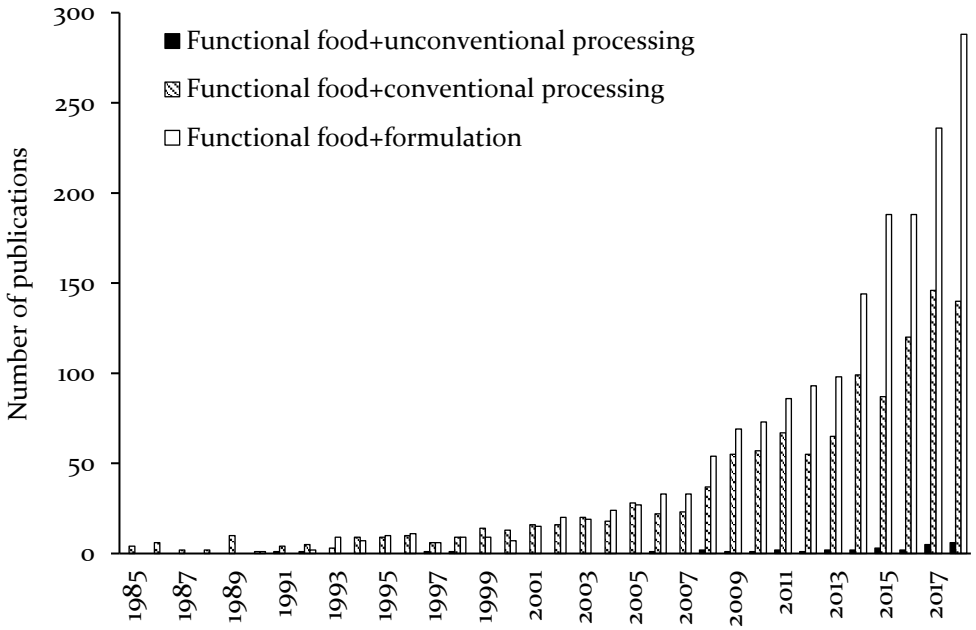


Figure 3. Number of papers published between 1985 and 2018 containing the keyword "functional food" associated with "unconventional processing", "conventional processing" or "formulation".

The general belief is that processing leads to an increase in food digestibility with respect to macronutrients, joint with a depletion in micronutrients and a consequent reduction in the nutritional value (Prochaska, Nguyen, Donat, & Piekutowski, 2000). This represents a drawback in the light of the major drivers for functional food design, namely reducing macronutrient uptake while increasing micronutrient delivery.

As a result, starting from 2006 a steep increase in functional food research driven by a formulation approach was observed, whereas matching "functional food" with "conventional processing" in the literature search almost halved the number of published papers on the topic (Figure 3). Even fewer studies have been carried out considering functional foods as affected by unconventional processing, indicating that, despite the interest in functional food development, researchers did not focus on the effect of different processing on food functionality, keeping the focus on the development of functional ingredients by a formulation approach. Thus, further research is indeed required to investigate the actual impact of technological interventions, considering both formulation and processing, as well as the co-ingestion with other foods and/or drugs, on the fate of bioactive compounds in real food matrices.

1.3.3 Evaluation

The success of a newly developed product is affected by several factors, including the carried health benefit, the market positioning, the communication, without neglecting the typical quality markers of a food product, such as the taste, convenience, and price (Kaur & Das, 2011).

As anticipated in paragraph 1.3.1, to authorize the commercialization and to claim the bioactivity of functional food, it is necessary first to demonstrate its efficacy (Reg. (EU) n. 1924/2006). This requires the collection of evidence and their submission to the EFSA, which provides a scientific opinion and eventually allows the use of specific nutritional or health claims.

If nutritional claims are approved based just on the nutrient content and the comparison with other products (Reg. (EU) n. 353/2008), the approval of health claims is more restrictive. In particular, these need to be based on generally accepted scientific evidence (Art 13.1, Reg. (EU) n. 353/2008) or on newly developed scientific data (Art 13.5, Reg. (EU) n. 353/2008), especially when dealing with the reduction of disease risk and referring to children's development and health (Art. 15, Reg. (EU) n. 353/2008). To obtain the approval it is thus necessary to present within the application the totality of available data. These

include human intervention/efficacy studies, randomized controlled trials, and human observational/epidemiological studies, possibly supplied with national and/or international expert consensus reports. Animal and *in vitro* studies are only accepted as supportive evidence of the mechanism underlying the beneficial effect for health but are not accepted alone. Moreover, the traditional knowledge and history of use, contrarily to what recently reported in Reg. (EU) n. 2015/2283 for the approval of novel foods from third countries, is not considered as valuable evidence for the authorization of health claims (Turck *et al.*, 2017).

Thus, evidence can be collected by applying the same approach as that used for orally ingested drugs, such as *in-vitro* mechanistic analysis, followed by animal and human studies. However, the behavior of a food matrix is definitely different as compared to that of orally ingested drugs. Even if they follow the same metabolic pathway, including liberation, absorption, distribution, metabolism, and excretion, applying the pharmacokinetic concepts to food is challenging. In fact, drugs contain one or few pure components targeting a specific action site, whereas food contains a plethora of compounds potentially interacting one with each other, thus resulting in a modified outcome (Motilva, Serra, & Rubio, 2015). In addition, these compounds are present in a complex food matrix mainly constituted by carbohydrates, proteins, and lipids that may affect the availability of bioactive compounds by different mechanisms (Parada & Aguilera, 2007). Nonetheless, the digestion process and the presence of a complex microbiota at intestinal level can modify these compounds thus affecting their bioactivity (Rein *et al.*, 2012). As a result, unraveling the efficacy of functional foods is challenging and pieces of information regarding the effects of food matrix on the bioactivity of some nutrients are still conflicting. The efficacy assessment remains thus an open issue that needs to be urgently resolved.

To explore and determine the mechanisms of action of bioactive compounds towards disease prevention, it is crucial to take into account all the steps building up the journey of bioactive compounds from the food matrix to the target tissue or organ where they can exert their health-promoting effect. These include the consumption, the gastrointestinal events, the absorption and the distribution (Motilva *et al.*, 2015). Understanding the factors governing the release of bioactive compounds from the food matrix and their extent of absorption and fate in the organism is thus essential (Cadenas & Packer, 2002). To this purpose, the key indicators to be considered are bioaccessibility, transformation, absorption, biodistribution, bioactivity, and bioavailability (Stahl *et al.*, 2002). These are defined as:

- ✓ **Bioaccessibility**: the fraction of ingested compound that is released from the food matrix thus becoming available for uptake by the intestinal mucosa (Ferruzzi, 2010). For hydrophilic molecules, it simply refers to the fraction of ingested bioactive that is dissolved within the gastrointestinal fluids, whereas for hydrophobic bioactive compounds it is typically taken to be the fraction that is incorporated within the mixed micelles formed in the small intestine (McClements *et al.*, 2015). It depends thus not only on the liberation, i.e. the release from its original environment, the food matrix but also on the solubility in the gastrointestinal vehicles and on the interactions occurring with other constituents present in the gastrointestinal tract.
- ✓ **Transformation**: bioactive compounds may incur in chemical (e.g. oxidation, reduction, hydrolysis) or biochemical (e.g. enzymatic metabolism) transformations within the gastrointestinal tract which may alter their molecular structure (McClements *et al.*, 2015).
- ✓ **Absorption**: it refers to the passage of bioactive compounds across the mucus layer, through the epithelium cells, and into the systemic circulation. It mainly depends on the permeability of gut wall and the mechanism of transport (active, passive, tight junction) as well as on eventual efflux mechanisms bringing back the compounds at gastrointestinal level (McClements *et al.*, 2015; Rein *et al.*, 2012).
- ✓ **Biodistribution**: it accounts for the temporal and spatial distribution of the bioactive compounds to different tissue compartments after intestinal absorption (Motilva *et al.*, 2015; Muss, Mosgoeller, & Endler, 2015). It mostly depends on the affinity of bioactive compounds for blood transport proteins, among which serum albumin is the most important. Although its primary physiological function is to carry hydrophobic fatty acid molecules around the bloodstream, it is the main carrier for drugs and for a number of other bioactive compounds deriving from food, such as polyphenols (Sengupta & Sengupta, 2003).
- ✓ **Bioactivity**: it stands for the physiological effect carried out by a bioactive compound once it has reached its site of action (Stahl *et al.*, 2002).
- ✓ **Bioavailability**: it represents the fraction of an ingested bioactive compounds that eventually ends up in the systemic circulation reaching the site of action in an active form to exert its bioactivity. It depends thus on all previously described physiologic pathways, and can be expressed as (Heaney, 2001; McClements *et al.*, 2015; Muss *et al.*, 2015; Stahl *et al.*, 2002):

$$Bioavailability = Bioaccessibility \times Transformation \times Absorption \times Biodistribution \times Bioactivity$$

To measure these indicators, both *in vitro* and *in vivo* studies can be applied. However, when assessing functionality, it is essential to keep in mind that some bioactive compounds do not need to enter to the systemic circulation to exert their bioactivity, as they may play an health-protective role already in the gastrointestinal tract (Motilva *et al.*, 2015). Thus, it is not always necessary to assess absorption and biodistribution.

Another critical aspect to be considered is the behavior of bioactive compounds, which may differ when they are included in a food matrix as compared to what occurs when they are tested as single molecules (Motilva *et al.*, 2015).

Thus, as reported in Figure 4, to assess food functionality it is recommended first to carry out preliminary evaluations through *in vitro* experiments, by simulating digestion.

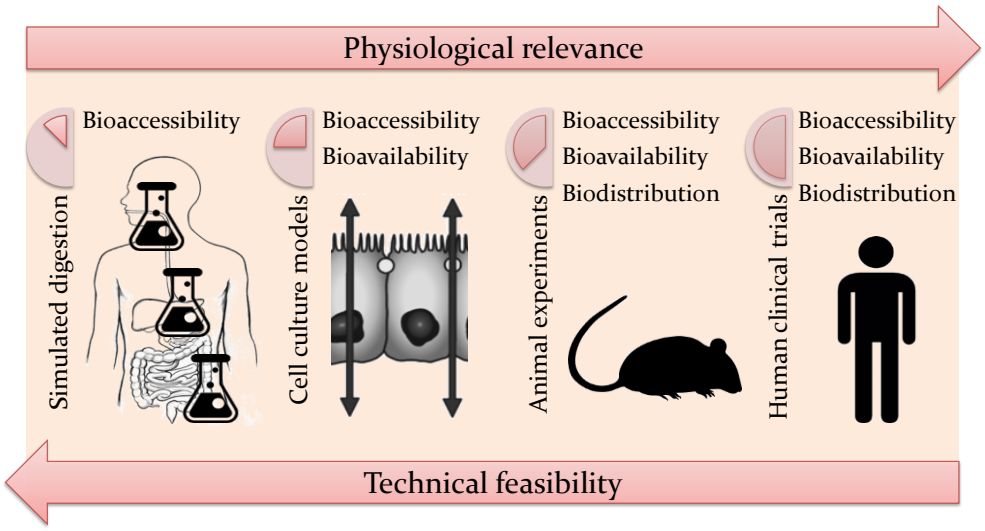


Figure 4. Methodologies for functionality evaluation progressively approaching physiological relevance, and related indicators.

Following, cell cultures can be used to mimic more closely biological conditions. These preliminary trials allow studying the matrix effect on food functionality. In addition, *in vitro* models carry several advantages, such as speed and excellent cost/effectiveness relation. However, their outcomes cannot always be directly extrapolated to *in vivo* systems. Thus, to get an insight into what actually occurs under physiological condition, *in vivo* preclinical trials using experimental animals, such as rats or mice, should be carried out. Although the outcomes deriving from these models may give an insight into the actual functionality under physiological conditions, they still do not allow to accurately determine human bioavailability; therefore, human testing still represents the gold standard (Gerster, 1999). This is essential to determine the dosage of bioactive compound leading a certain effect and to assess the efficacy of a food vehicle in delivering such functionality. In fact, according to the technical guidance for the preparation and presentation of an application for

authorization of a health claim, *in vitro* and animal experiments are only accepted as supporting data (Turck *et al.*, 2017), whereas human clinical trials are compulsorily required. The requirement for *in vivo* substantiating brings about the hindrance represented by such studies: they are costly, time-consuming, complex to manage and ethically constraining (Brodkorb *et al.*, 2019), as compared to *in vitro* experiments (Figure 4). It is thus essential to get as much information as possible from the *in vitro* experiments, in order to select the most promising interventions to be validated through human clinical trials.
To do so, it is necessary to develop and apply standardized methodologies able to assess the expected health benefit, to identify the bioactive compounds responsible for such benefit, and to determine their mechanism of action.

1.3.3.1 Simulated digestion

In vitro models have been used for many decades to simulate the digestion of food, while overcoming the hindrances of *in vivo* studies.
In vitro digestion models can be divided into static and dynamic methods. Although dynamic methods are more physiologically relevant, they are highly complex, require substantial hardware and software and are expensive to set up and maintain (Dupont *et al.*, 2018). Thus, the majority of food researchers apply the static ones, owing to their simplicity, good *intra-* and *inter*-laboratory reproducibility, robustness, relatively low cost (Minekus *et al.*, 2014). These methods mimic the three successive digestive phases, namely the oral, gastric and intestinal, during which the experimental conditions (i.e. ratio of food to enzymes and electrolytes, pH, T, time) are kept constant.
Although standardized static models have been developed to be applied in the pharmaceutical field (United States Pharmacopeia methods), these resulted unsuitable for digesting food because of the complexity and variability of food structures. Thus, a great number of different digestion methods have been applied in the food sector, all exhibiting different conditions and making a comparison between studies impossible. The need for a harmonization of digestion conditions led to the international INFOGEST network (http://www.cost-infogest.eu) to build an international consensus on a set of digestion parameters, with particular attention to the enzyme activity, for static *in vitro* simulation of digestion suitable for food. The method was published in 2014 (Minekus *et al.*, 2014) and is now increasingly applied by researchers all over the world. Based on the first results and identified gaps, update and improvement of the procedure have recently been published (Brodkorb *et al.*, 2019).

The increase in precision and reproducibility guaranteed by the standardized method makes it a useful tool for sample screening. Still, only a few studies have evaluated the *in vitro–in vivo* correlation, highlighting the urgent need for more research on this topic so that more realistic static *in vitro* models can be developed (Hur, Lim, Decker, & McClements, 2011).
However, it should be kept in mind that the impossibility of mimicking the complex dynamics of the digestion process (i.e. the pH gradients, the gradual addition of enzymes and gastric fluid, the continuous gastric emptying) as well as the physiological interactions with the host (i.e. the absorption and biodistribution kinetics) limits the ability of static *in vitro* digestion in predicting the fate of food bioactive compounds during gastrointestinal digestion (Dupont *et al.*, 2018). Thus, applying this model can only provide indications relevant to bioaccessibility and transformation, whereas no insights regarding the absorption and biodistribution can be gathered.
Still, it represents a key step to pre-qualify the most promising technological interventions able to boost food functionality. It is thus essential to have available a reliable tool for a preliminary assessment of food functionality before applying more complex models.

1.3.3.2 Cell culture models

In some cases, cell cultures are used as part of *in vitro* digestion models. In particular, the Caco-2 cell culture model has been widely applied to predict the absorption of bioactive components from foods and pharmaceutical preparations (Hur *et al.*, 2011). These models allow investigating the mechanism and extent of absorption of bioactive compounds, indicating the ratio of compounds that besides being bioaccessible, can also be delivered through blood vessels to the target organs and tissues (McClements *et al.*, 2015). Cell culture models may also provide indications relevant to metabolic reactions occurring in the gut lumen or within the enterocyte, due to the presence of brush-border and intracellular enzymes, and resulting in the modification of bioactive chemical structure before absorption (Kern *et al.*, 2003; Konishi & Kobayashi, 2004).
However, for most bioactive compounds the metabolic pathway has not been fully characterized yet. This represents an essential step to understand which are the compounds responsible for the functionality of food and in which form do they carry out the health-promoting effect (Rein *et al.*, 2012). Having a more complete picture of the circulating bioactive metabolites could help in defining the technological interventions able to steer food functionality and enhance bioavailability.

1.3.3.3 *In vivo* trials

After the preliminary *in vitro* screening, it is necessary to switch to *in vivo* models to understand the actual physiological response to specific foods. To follow the complex digestive processes within the digestive tract in detail, animal studies mostly involving rats and pigs have been carried out. Although these animal models allow getting as close as possible to physiological human conditions, their relevance for understanding food digestion in humans has been questioned. In addition, their use generally involves animal death or surgical approaches in which cannulas are placed into digestive organs to access the contents of the gastrointestinal tract, being thus not always justifiable on ethical grounds (Brodkorb *et al.*, 2019).

As previously reported, human interventions represent the gold standard model to study food functionality (Gerster, 1999). However, also these studies require invasive procedures, such as aspiration and sampling of digestive effluents from the stomach or small intestine, by means of nasogastric or nasointestinal probes, bringing about ethical issues (Sullivan *et al.*, 2014). To overcome this shortcoming, ileostomy patients have been proposed and used to study digestion (Kahle, Kraus, Scheppach, & Richling, 2005), but they cannot be considered as models of a healthy human, as they are generally affected by digestive pathologies. Less invasive techniques can alternatively be applied, such as imaging technologies and wireless telemetric systems (Sullivan *et al.*, 2014), but no insights into the mechanisms leading to a health-promoting effect upon the consumption of a certain food can be gathered by these approaches.

Similarly, although human intervention trials have often been used to correlate the consumption of a certain food product with a health-promoting effect, they can only provide indications about the efficacy of tested food towards the prevention of a certain disease but do not give insights into the mechanism underlying the functional properties.

Thus, even if human trials are compulsorily required to claim food functionality (Reg. (EU) n. 353/2008), human models can only represent the final step of efficacy validation (Dupont *et al.*, 2018). Other models, providing details about the molecules carrying bioactivity and the mechanism by which such effect is provided are essential to steer technological interventions in order to drive functionality.

1.3.3.4 Measuring the bioactivity

Both *in vitro* and *in vivo* trials are carried out to get an output which can be related to the physiological effect of the ingested functional food, as the interest towards functional foods lies in their ability to manage or prevent several chronic diseases. Food functionality has been generally attributed to bioactive compounds principally having antioxidant, anticarcinogenic and antidiabetic abilities. These properties have been tested by applying several techniques, but most of them consider *in vitro* trials, with deriving advantages and drawbacks.

As already pointed out, *in vitro* tests are easy, cheap and fast. However, they do not consider the complexity of biochemical conditions occurring in the human body.

For instance, the antioxidant capacity has commonly been tested by determining the ability to scavenge radical species (Boyer & Liu, 2004; Nicoli, Anese, & Parpinel, 1999). Different methods are available, and results may differ depending on the oxidant/reductant used, as well as the compositional and environmental variables. For instance, the ABTS assay is based on the reduction of the antioxidant due to the loss of an electron by the nitrogen atom of ABTS, whereas the FRAP method relies on the oxidation of the antioxidant leading to the reduction of the complex ferric ion-TPTZ (Pisoschi & Negulescu, 2012). As the response of antioxidants to different conditions differs, it should be kept in mind that claiming the antioxidant efficacy in a complex system, such as food, generally requires to test this property by applying more than one technique, in order to avoid result misinterpretation (Prior, Wu, & Schaich, 2005). Still, several authors recently questioned the biological relevance of *in vitro* results relevant to the antioxidant capacity of bioactive molecules, as these assays do not consider physiological conditions (Fawole & Opara, 2016; Ludwig, Clifford, Lean, Ashiharad, & Crozier, 2014). In addition, in recent years the hypothesis of a direct connection between health effects and antioxidant activity has been debunked (Del Rio *et al.*, 2013; Song, Manson, Buring, Sesso, & Liu, 2005). Other mechanisms were suggested to explain the health-related properties of food bioactive compounds, with particular attention to the phenolic ones.

Many researchers are currently focusing on the antidiabetic effect of polyphenols. This depends on their ability to inhibit amylolytic enzymes involved in carbohydrate digestion pathway (Agustinah, Sarkar, Woods, & Shetty, 2016; Tadera, Minami, Takamatsu, & Matsuoka, 2006), resulting in a reduced release of glucose at intestinal level and thus in a reduction in blood glucose level after the meal. These enzymes are targeted by several antidiabetic drugs (Akkarachiyasit, Charoenlertkul, Yibchok-Anun, & Adisakwattana, 2010).

Thus exactly identifying the mechanism by which phenolic compounds produce an antidiabetic effect could help in designing dietetic tailor-made interventions potentially reducing drug dosage and thus the related side effects such as weight increase, hypoglycemia and gastrointestinal diseases (Cheng & Fantus, 2005). Also in this case, *in vitro* trials are the most widely applied techniques to test the inhibitory capacity of phenolic compounds against amylolytic enzymes (Nasu, Miura, & Gomyo, 2005; K. Singh *et al.*, 2014). These trials allow getting detailed knowledge about the inhibition mechanism (E. M. Ahmed, El-maraghy, Teleb, & Shaheen, 2014). Still, as in the case of antioxidant activity, they hardly mimic relevant physiological conditions.

Thus, *in vivo* testing results essential to assure the efficacy of technological interventions aimed at producing functional foods. The biological activity is generally measured *in vivo* by monitoring the parameters of interest relevant to a specific disease, through direct and dynamic measurements (Heaney, 2001). For instance, the antidiabetic efficacy can be assessed by measuring the glycemic response during the time after food consumption (Wolever, Jenkins, Jenkins, & Josse, 1991). Similarly, triglyceride blood levels are informative about interventions aimed at reducing lipid absorption (S.-Y. Tan, Wan-Yi Peh, Marangoni, & Henry, 2017). Still these tests are not informative about the mechanisms underlying the observed effect.

Due to the need to understand both the efficacy under physiological conditions as well as the mechanism behind food functionality, there is an increasing interest into converging the disciplines dealing with the monitoring, modeling and analysis of complex biological systems. To this regard, the development of wearable devices has experienced a steep increase (US 9189021 B2, 2015; US 6513532 B2, 2003; US 8073707 B2, 2011), as these are becoming popular to monitor continuously the health status (Piwek, Ellis, Andrews, & Joinson, 2016). These devices give direct access to personal analytics that can contribute to facilitating preventive care and aid in the management of ongoing illness. For chronic food-related diseases, wearables could be applied as a novel tool to assess food functionality *in vivo*, obtaining real-time detailed data, without involving more sophisticated, uncomfortable, and expensive alternatives (Piwek *et al.*, 2016). In particular, some of these systems can be inserted in the oral cavity to measure food intake for controlling eating behavior and to provide data directly related to chronic diseases such as hypertension, diabetes, and obesity (Lee *et al.*, 2018). In this way, patient progress could be monitored to assess the efficacy of applied interventions, which in turn can be personalized, thanks to the emerging scientific field of nutrigenomics (German, Zivkovic, Dallas, & Smilowitz, 2011).

Although several studies have attempted automating dietary monitoring using wearable devices, several issues remain opened (Prioleau, Ii, Member, & Paper, 2017). Few studies are available on the reliability of acquired data, as the major hindrance lies in the interference of signals. In addition, the output interpretation depends on the understanding of the analyzed biological system, requiring a deep knowledge of the mechanisms underlying targeted functionality.

1.3.4 Marketing communication

The success of a newly developed functional food is affected not only by the carried health benefit but also by several other factors, such as market positioning and communication, without neglecting the typical quality markers of a food product, such as the taste, convenience, and price (Kaur & Das, 2011). Thus, the efficacy assessment cannot be strictly related to the functionality aspect but should take into account an effective communication of the benefits carried by the food, in order to deliver a successful product on the market.

Settling the most suitable communication strategy requires to consider many determinants affecting consumption intention and the subsequent decision to accept or reject a new food (Giordano, Clodoveo, De Gennaro, & Corbo, 2018). To make a decision of food consumption, humans rely on two coexisting and opposite tendencies: the curiosity towards novelty and the consequent tendency to try new and unfamiliar foods, also called *neophilia*, and the prudence with respect to new products perceived as being potentially dangerous, namely *neophobia* (Fischler, 1988).

Communication plays a key role in the decision to accept/reject newly developed foods, as it should find the optimal strategy, able to induce curiosity towards novelty while preventing fear (Giordano *et al.*, 2018). To do this, it is essential to know the drivers leading to consumption intention and decision. Consumer and product features represent the primary factors affecting consumer behavior (Giordano *et al.*, 2018).

Consumer characteristics include gender, age, and knowledge about health and diet relationship. It is noteworthy that the latter is not necessarily in line with actual benefits. In addition, consumers aware of health-related benefits represent a niche of the targeted market, making thus necessary efforts to reach also the other consumers.

Product features can be further distinguished into intrinsic attributes, such as the presence of health-promoting compounds, and extrinsic ones, including package, brand and label claims (Bimbo *et al.*, 2017).

Still, research is mostly focused on product intrinsic features and product development is most commonly driven by technical feasibility. As a result, a mismatch between the features of newly developed functional foods and consumer expectations and needs may occur (Van Kleef, Van Trijp, & Luning, 2005). Consequently, there is a high risk of product failure (Bleiel, 2010), with 70 to 90% of new health-enhancing products withdrawn from the market within the first two years from their launch (Research and Markets, 2015).

This depends on the fact that, besides intrinsic product features, both its extrinsic attributes and the consumer characteristics account for a considerable effect on the intention to consume a product. Among the extrinsic attributes, labeled information may substantially contribute to the success of new functional food. Health claims can only give true added value to the product if consumers both recognize the benefit and find it important (Lähteenmäki, 2013). However, claims are often formulated in complicated terms: shorter and easier to understand claims may increase the efficacy of the communicated benefit and thus push the consumption intention (Siegrist, Stampfli, & Kastenholz, 2008). Still, this is not affected only by the primary factors, i.e. product and consumer characteristics, but is the result of their matching with the secondary ones, which are represented by **risk perception**, **subjective norms** and **cost/benefit balance**, with the latter playing the most critical role. This takes into account both individual benefits, such as healthiness, convenience, sensory aspects, and advantages for the society, such as sustainability improvement, food security, facilitation of healthier lifestyles (Giordano *et al.*, 2018).

In the light of these considerations, the complexity and the huge number of factors and mechanisms behind consumption intention and decision is outstanding. Since getting an insight into these aspects is crucial for manufacturers to successfully deliver the product on the market, consumer research is applied during testing and launching of a new product (Van Kleef *et al.*, 2005).

In particular, the efficacy of nutrition and health claims can be assessed through different tools, such as conjoint analysis, category appraisal, focus group, free elicitation, among others (Van Kleef *et al.*, 2005). Although the availability of many tools to assess consumer reaction to claims, literature studies report contradictory results. For instance, according to several authors (Bimbo *et al.*, 2017; Van Kleef *et al.*, 2005) consumers prefer specific health claims, dealing with the prevention or risk reduction of a disease (e.g. heart failure) more than general nutritional ones, such as those dealing with wellbeing (e.g. energy-boosting). On the contrary, Schnettler *et al.* (2019) reported that consumers find generic nutrition labeling schemes more useful than specific health claims. Such contradictory results may

depend on the different decision patterns applied to different product categories (Bimbo *et al.*, 2017), suggesting the need for a tailor-made product assessment.
The results deriving from these investigations provide an essential tool to implement an approach to innovation that is not just product-driven but consumer need-driven instead (Van Kleef *et al.*, 2005).
Actually, different approaches to drive innovation already exist, but those with the lowest consumer involvement are still the most common, requiring his/her intervention only in the prototyping and launching phases and disregarding the crucial role he/she could potentially play in the first phases, namely idea generation and concept design (Busse, Siebert, Busse, & Siebert, 2018).
Deeper involvement of consumers within the functional food development cycle would not only provide indications to develop customized and effective communication strategies but could result in knowledge dissemination.

1.3.5 Dissemination

As anticipated (paragraph 1.3), dissemination is essential to merge each step of the functional food development cycle and to guarantee its effective functioning (Figure 2).
In fact, it represents the process of making available knowledge to stakeholders and to the widest audience. Dissemination can thus be intended as a cross-cutting activity which can be decoupled into two aspects.
On the one hand, it is intended as informing and educating the general public about the functional food issue. Nowadays, the "hot topics" to deal with in the food field are nanotechnologies, genetically modified organisms, nutrigenomics, irradiation, animal cloning (Giordano *et al.*, 2018). Overall, consumers demonstrate a high awareness, but a low level of actual knowledge about these matters. This leads to high levels of concern, resulting in hesitation in accepting functional foods especially when obtained by applying novel technologies (Siegrist, 2008). As consumer attitude towards food innovation is not only affected by the innovation itself, but also by the surrounding environment (Henson, 1995), it is essential to carry out dedicated actions to increase the actual knowledge about the benefits associated with the consumption of newly developed functional foods (Bech-Larsen & Scholderer, 2007). It is worthy to note that educating consumers about food functionality issues is crucial because, unlike taste and other sensory traits, consumer cannot perceive directly the benefits of the product (Siró *et al.*, 2008). These actions may include educational activities in the schools, as well as awareness-raising campaigns in public places. To increase

the trust of public towards provided information, these activities should be carried out by independent scientists or consumer organizations, which are considered to share similar values with the consumers (Siegrist, 2008). Despite the importance of information coming from a trusted third-party source, as addressed more in detail in paragraph 1.3.4, another important channel to increase consumer awareness is the labeled information (Lähteenmäki, 2013), even though this is provided by the manufacturer himself.

The other aspect relevant to dissemination refers to raising awareness and engage stakeholders. It relies on the exchange of information about the ongoing research and current development in the functional food field among professionals, such as scientists and researchers, health professionals, nutritionists, food technologists (Gur *et al.*, 2018). Dissemination can be carried out by conventional ways, such as conferences, workshops, and scientific publications, but also through emerging channels, including the social network (Cooper, 2014). The required effort is to find a common language among experts involved in the functional food development cycle at different levels, to draw an exhaustive picture on the state of the art and to identify the most effective strategy to be followed in the development of functional foods.

1.3.6 Actors involved in the functional food development cycle

Based on previous considerations and as in general defined for the innovation systems by Freeman (1987), also the development of functional food is a non-linear, multiple-actor process. The different subjects involved present different interests, perspectives, and skills, which need to be merged into a network able to produce an integrated outcome (Moors, 2012). Table 3 reports the main actors involved in the functional food development cycle.

Table 3. Actors and roles within the functional food development cycle.

		Step			
	Actor	Definition	Design	Evaluation	Communication
Divulger	Clinician			✓	
	Legislator	✓			✓
	Nutritionist		✓	✓	
	Food technologist		✓	✓	
	Marketing expert				✓
	Consumer		✓	✓	✓
		Dissemination			

As functional food consumption is associated with a health-related benefit, clinicians are expected to be one of the first figures dealing with the consumer needs with respect to

functional foods (Bigliardi & Galati, 2013). A chronic disease has to be tackled not only through proper pharmacological intervention but requires also changes in the dietary habits (Mansukhani, Volino, & Varghese, 2014; P. K. Prabhakar, Kumar, & Doble, 2014). In these terms, the clinical doctors are responsible for dissemination both to the general public and to the experts. As discussed in paragraph 1.3.5, the former is mostly related to providing the patients with information about functional food health-protective potential. In these terms, they play a crucial role as they are trusted by consumers, being a third-party source (Lähteenmäki, 2013). Dissemination to experts is intended as the disclosure of regulatory requirements to the legislator (Gur *et al.*, 2018). It also refers to displaying to nutritionists and food technologists the needs to be accomplished when designing new foods. Clinicians are then involved in the evaluation step when dealing with human clinical trials.

The legislator outlines and implements policies to influence the innovation process (Moors, 2012). To do so, he collects technical information from the other actors involved in the cycle and lays down definitions and regulation, accordingly. In this way, he guarantees the operability of the functional food market. He also lays down the rules to claim health benefits deriving from functional food consumption. In doing so, he is also responsible for effective communication to the consumer and more in general for the dissemination activity (Siró *et al.*, 2008).

The nutritionist cooperates with the clinicians for the dissemination to the general public with respect to food-related health issues. He is responsible for defining nutritional needs and driving consumer choices (Rodríguez-Cerezo, Stein, & Rodríguez-Cerezo, 2008). He disseminates technical information to the legislator, with particular reference to nutritional claims (Arboleya, Lasa, Olabarrieta, & Martinez de Maranon, 2010). He also disseminates his knowledge to experts and in particular to food technologist, playing an active role in the design step (Bigliardi & Galati, 2013). Afterward, he is also involved in the efficacy evaluation step relevant to human clinical trials, in cooperation with the clinicians.

The food technologist plays a crucial role in the functional food development cycle due to his technical skills and knowledge of the food matrix and the technological interventions (Bigliardi & Galati, 2013; Granato, Nunes, & Barba, 2017). His major task is thus the design of functional foods, but he is also involved in the efficacy evaluation. He can independently apply preliminary *in vitro* assessments to screen the most effective interventions. He cooperates then with nutritionists and clinicians to test newly developed food for their efficacy through cell culture models and *in vivo* trials (Granato *et al.*, 2017; Jones & Jew, 2007).

The food technologist also provides technical information to the legislator (Coppens *et al.*, 2006) and handles public education with respect to technological issues (Siegrist, 2008).
The marketing expert is mostly responsible for effective communication to consumers of food functionality through labeling, as long as claim regulation is accomplished (Siró *et al.*, 2008). He deals with consumer attitude and acceptability towards functional foods, with special attention to food neophobia issues and his know-how is essential to design market surveys (Damsbo-Svendsen, Frøst, & Olsen, 2017; Van Kleef *et al.*, 2005). His skills can also be useful during the dissemination step, to define the most effective strategies to drive consumer choices and increase their actual knowledge and awareness about functional food issues (Küster-Boluda & Vidal-Capilla, 2017).
Noteworthy, from the dissemination viewpoint, the clinician, the legislator, the nutritionist, the food technologist, and the marketing expert can all be regarded as divulgers (Table 3).
Although the innovation process generally relegates the consumer as a passive objective of observation, the literature reports on the important role that consumers may play in leading the innovation process at different stages (Busse *et al.*, 2018), determining the success of a newly developed functional food. Consumers also represent the observation subject when dealing with the communication step, providing indications about the most effective strategies (Van Kleef *et al.*, 2005).
As turns out from Table 3, a collaborative cross-network is required to deliver successful functional foods, with continuous participation of stakeholders at all stages (Busse *et al.*, 2018; Khan, Grigor, Winger, & Win, 2013). Nonetheless, the core topic of the functional food development cycle remains the design of the product, closely around followed by the evaluation of its efficacy. Thus, although many figures are involved in the cycle, food technologist and nutritionist, together with clinicians, play the most crucial role in functional foods development.

1.4 Holistic approach to design and evaluation

As design and evaluation represent the key technical steps in the development of functional food it is essential to merge them. However, this is not straightforward as it is difficult to find a common language between the actors involved in these steps.
As previously reported (paragraph 1.3.6), although role overlapping is desirable in many steps, food design is typically carried out by food technologists, while the assessment of functionality generally relies on the activity of nutritionists and clinicians. A gap between

the technological and the nutritional viewpoints can be identified by analyzing the current literature.

Some attempts have been made to match the complementary skills deriving from the different actors. However, when the technological viewpoint prevails, the physiological effect (i.e. digestion) is not considered. As a result, the targeted bioactivity is evaluated by applying *in vitro* trials considering the undigested food matrix.

Conversely, when the nutritional viewpoint prevails, the effect of digestion on bioactive compounds is assessed by applying both *in vitro* and *in vivo* studies mostly on simplified model systems, thus not considering the technological history of food.

This approach seems disjoint as it does not take into account the complexity of the food matrix, the potential interactions occurring among food components, the effect of technological interventions, nor the actual bioactivity upon digestion. To bridge this gap, a holistic approach to design functional foods and assess their functionality is here proposed (Figure 5).

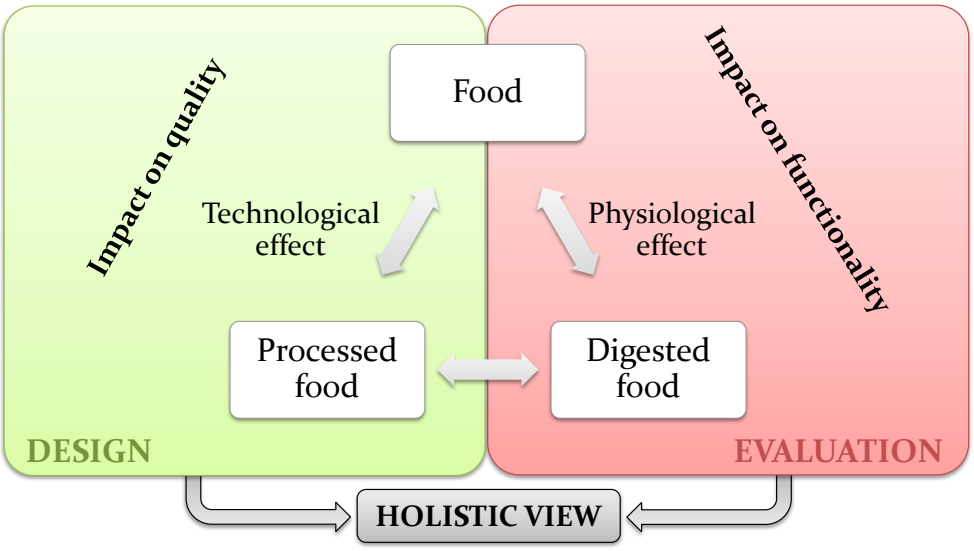


Figure 5. Holistic approach to design functional foods and assess their functionality.

Based on this approach, the technological effect is first assessed in terms of impact on the safety and quality of food. This focuses on the physical, chemical and sensory properties. It should be kept in mind that consumers are not willing to compromise the taste on behalf of health benefits (Lähteenmäki, 2013). It is then essential to meet consumer expectation when designing functional food. Technological interventions should be carried out to concomitantly booster food functionality while maintaining or improving product quality. Once the newly developed product satisfies quality requirements, the impact on functionality needs to be assessed, by measuring the bioavailability and comparing it among

different technological interventions. It is then crucial to identify the best compromise between quality and functionality.

Indeed, matching the outcomes deriving from the technological and the nutritional evaluations would allow assessing the efficacy of applied interventions on food functionality from a holistic viewpoint.

1.5 Potential application of functional foods: the case of type 2 diabetes

During the last century dietary habits have considerably changed, leading to an excessive consumption of highly refined sugars and high-energy-density foods. Such dietary change, together with a sedentary lifestyle, has been correlated with the increasing incidence of chronic metabolic diseases. Among these, one of the most alarming is type 2 diabetes mellitus. It accounts for approximately the 90% of diabetes cases, while the 10% is represented by type 1 and gestational diabetes. It is expected to become the 7th death cause by 2030, affects 1 out of 10 people worldwide and accounts for 12% of overall healthcare costs (WHO, 2016). Besides these directly tangible costs, it represents also an indirect financial burden, causing more than 200 million €/year loss in economic growth, due to a decreased efficiency, missed working days and early retirement (WHO, 2016).

Type 2 diabetes is a non-communicable metabolic disease characterized by hyperglycaemia within a clinical situation of insulin resistance and relative insulin deficiency (Kumar *et al.*, 2005). The major risk factors for type 2 diabetes are physical inactivity, unhealthy diet, overweight and obesity, especially when associated with a genetic disposition. In other words, three out of four risk factors are connected with diet. Actually, in the last decades, diabetes rate has grown at the same pace of obesity incidence.

Although the administration of drugs may in some cases become necessary (Mazze *et al.*, 1984), due to side effects associated with their intake (Pranav Kumar Prabhakar & Doble, 2011), the need for alternative solutions has become impellent (Cheng & Fantus, 2005; Ríos, Francini, & Schinella, 2015).

To this regard, dietary changes have been suggested to limit type 2 diabetes occurrence (American Diabetes Association, 2004). Thus, in the last years the research interest towards the development of functional foods able to manage type 2 diabetes has undergone a steep increase.

Several foods, especially those of vegetable origin, seem promising in terms of antidiabetic properties and are currently under investigation. The antidiabetic effect has mostly been attributed to the presence of bioactive compounds, which have been observed to modulate the glycemic response of foods (Ali *et al.*, 2016; Mansukhani *et al.*, 2014).

According to the literature, one of the foremost mechanisms by which bioactive compounds elicit an antidiabetic effect is the inhibition of α-glucosidase (Ali *et al.*, 2016; Lebovitz, 1997). This is a hydrolase located on the intestinal cell membrane of the ciliate epithelium and is

required to obtain glucose from oligo- and disaccharides (Chiba, 1997). α-Glucosidase plays thus a key role in the final step of carbohydrates digestion and its inhibition currently represents a common therapeutic approach to reduce postprandial hyperglycemia (Goto *et al.*, 2012; X. J. Hu, Wang, & Kong, 2013; Kwon, Apostolidis, & Shetty, 2008). In fact, the inhibitory effect is conventionally obtained by the so-called glycomimetic drugs (i.e. acarbose, voglibose, miglitol) that can interact with the enzymatic active site (Akkarachiyasit *et al.*, 2010). Nonetheless, these drugs induce weight increase, excessive hypoglycemia and gastrointestinal diseases (Cheng & Fantus, 2005).

The bioactive compounds able to slow down the activity of α-glucosidase, thus retarding carbohydrate digestion and glucose absorption, are mainly phenolic compounds (Xiao & Högger, 2015), such as chlorogenic (Iwai *et al.*, 2012) and cinnamic acids (Adisakwattana, Chantarasinlapin, Thammarat, & Yibchok-Anun, 2009), catechins (Geng, Shan, Ma, & Liu, 2016) and kaempferol (Peng, Zhang, Liao, & Gong, 2016).

Besides directly inhibiting α-glucosidase thanks to the presence of bioactive compounds, vegetable matrices also contain dietary fiber, which can also contribute to reducing the glycemic response by forming a gel able to entrap starch granules, in the case of the soluble fraction, and by increasing matrix viscosity, in the case of the insoluble fraction, hindering in both cases amylolytic enzyme activity (Brennan, 2005; Juvonen *et al.*, 2009; Wilfart, Montagne, Simmins, Noblet, & van Milgen, 2007).

Based on these considerations, promising approaches in the development of functional foods aimed at managing type 2 diabetes are on the one hand to boost the antidiabetic capacity of foods already presenting this property and on the other hand to use functional ingredients rich in bioactive compounds in the formulation of commonly consumed foods to reduce their glycemic response.

However the literature relevant to the effect of technological interventions, intended as formulation and processing, on food antidiabetic potential is not exhaustive and provides uncertain indications (Brennan, Blake, Ellis, & Schofield, 1996; Ferrer-Mairal *et al.*, 2012).

Aim and outline of this Ph.D. thesis

This Ph.D. thesis aimed at investigating the effect of food design, carried out by different technological interventions of formulation and processing, on the antidiabetic potential of several foods, known for containing bioactive compounds. Their antidiabetic effect was further assessed as affected by the combination with drugs or with other foods. The evaluation of the antidiabetic potential was performed by applying both *in vitro* and *in vivo* trials.
The study cases considered in the present Ph.D. thesis, the design actions and the evaluation activities are reported in .

In **Chapter 2** turmeric was considered as a bioactive ingredient.
It was used to enrich sunflower oil, which was structured by using different oleogelators. Lipolysis kinetics and curcuminoid bioaccessibility as affected by structuring were assessed upon *in vitro* digestion.

Chapter 3 reports the study case relevant to coffee.
The first part considered the effect of coffee roasting on *in vitro* α-glucosidase activity, by evaluating the inhibition extent and the mechanism of action.
In the second part, the effect of roasting on the antidiabetic potential of coffee was further explored considering *in vitro* digestion.
The third part examined how the addition of milk with different fat content and the application of high-pressure homogenization affected chlorogenic acid bioaccessibility and α-glucosidase inhibitory capacity of coffee.

In **Chapter 4**, the case of apple juice was investigated.
In the first part, the effect of thermal pasteurization on *in vitro* α-glucosidase inhibitory activity of apple juice was assessed. The interaction with acarbose was also explored by *in vitro* tests and in healthy rats.
The second part aimed at further studying the effect of thermal pasteurization on the potential bioactivity of apple juice as affected by *in vitro* digestion. Ultrasound pasteurization was also considered, and the bioavailability of phenolic compound was studied by using a cell culture model.

Chapter 5 deals with apple pomace, i.e. the processing by-product of apple juice industry.
In the first part, it was used as a functional ingredient to reduce the glycemic response of short dough biscuits. The glycemic response was assessed both by *in vitro* digestion and *in vivo* on healthy rats. These were also fed with conventional or reformulated biscuits co-administered with different beverages (i.e. a soft drink and apple juice) to test the concomitant effect of a formulation and a dietary intervention on the glycemic response. Consumer attitude was finally studied as affected by the communication of the reformulation intervention through labelled claims relevant to food functionality.

Table 4. Study cases, design actions and evaluation activities considered within the Ph.D. thesis.

	Design			Evaluation		
Study case	**Formulation**	**Processing**	**Diet/drug combination**	**Simulated digestion**	**Cell culture model**	**Animal experiment**
Turmeric 	Oil enrichment and structuring			Curcuminoid bioaccessibility		
Coffee 		Roasting		Phenolic compound bioaccessibility α-Glucosidase inhibition and mechanism		
	Milk addition	HPH		Phenolic compound bioaccessibility α-Glucosidase inhibition		
Apple juice 		Thermal pasteurization		Phenolic compound bioaccessibility α-Glucosidase inhibition Human serum albumin binding	Phenolic compound bioavailability	
		Ultrasound pasteurization		Phenolic compound bioaccessibility α-Glucosidase inhibition Human serum albumin binding		
			Acarbose	Interaction towards α-glucosidase inhibition		Glycaemia
Apple pomace 	Biscuit reformulation			Glycemic index		Glycaemia
			Soft drink			Glycaemia
			Apple juice			Glycaemia

Chapter 2 Turmeric

The rhizome of *Curcuma longa*, a plant of the *Zingiberaceae* family, is commonly used to produce the spice turmeric, whose medicinal use has been documented since ancient times in Ayurveda (the traditional Indian medicine). In recent years, turmeric functional properties have been investigated (Zou, Liu, Liu, Xiao, & McClements, 2015), and it turned out that curcuminoids, although accounting for less than 10% of turmeric weight, are responsible not only for its yellow color, but also represent its major bioactive compounds (Prasad, Gupta, Tyagi, & Aggarwal, 2014). Curcuminoids are represented by three major compounds: bisdemethoxycurcumin, demethoxycurcumin and curcumin (Shishu & Maheshwari, 2010). The latter is the most abundant molecule, representing between 80 and 90% of total curcuminoids, whereas demethoxycurcumin and bisdemethoxycurcumin account for the 6 and less than 1%, respectively (Shishu & Maheshwari, 2010).

Curcuminoids are responsible for multiple health-promoting effects (Aggarwal *et al.*, 2003), such as anticarcinogenic (Zheng *et al.*, 2014), anti-inflammatory (Prasad *et al.*, 2014), antioxidant activities (Jayaprakasha, Rao, & Sakariah, 2006) and hypoglycemic effects (Aziz *et al.*, 2013). The latter derives by several mechanisms, among which are the protection of pancreatic cell and the increase in the insulin sensitivity (Su, Wang, & Chi, 2017). The occurrence of these mechanisms has been ascribed to the presence of many chemically reactive functional groups, enabling it to participate in several biochemical reactions (Heger, van Golen, Broekgaarden, & Michel, 2014) with different molecular targets, including transcription and growth factors, protein kinases and cytokines (Zhou, Beevers, & Huang, 2011). In addition, no toxicity was reported for curcumin at the levels required to provide a beneficial health effect (Hatcher *et al.,* 2008).

Despite the beneficial health effects of curcuminoids, the major issue is related to their limited bioaccessibility. This depends on the poor water solubility and chemical instability, leading to reduced solubilization in the gastrointestinal aqueous fluids, with consequent low oral absorption (Anand *et al.*, 2007) and inefficient uptake by the epithelial cells (Kharat *et al.*, 2017).

Although the uptake and distribution of curcumin in body tissues is crucial to guarantee its biological activity, only a limited number of studies have addressed this issue (Perkins *et al.*, 2002; Sharma *et al.*, 2004; K. Y. Yang, Lin, Tseng, Wang, & Tsai, 2007).

To overcome the low bioaccessibility and bioavailability, a common strategy in the field of lipophilic bioactive compounds is to dissolve them into lipidic matrices. In the traditional Indian medicine, turmeric powder is actually administered orally as a dispersion in a mixture of clarified butter and milk or is consumed as ingredient in curry, by dispersing it in fatty cooking media (Shishu & Maheshwari, 2010). A further strategy is to use adjuvants, namely compounds boosting its bioavailability, such as piperine (Shoba *et al.,* 1998) and quercetin (Cruz-Correa *et al.,* 2006).

Still, these strategies do not provide enough protection against the gastrointestinal conditions. Other promising approaches are encapsulation (McClements, 2012) and inclusion in emulsified systems (Anand *et al.,* 2007). Besides preventing degradation during digestion, the latter can also improve the dispersibility of curcumin, thus resulting in a higher concentration of loaded compound. Emulsion-based delivery systems present several advantages for the application in the food industry: as many food products already exist in the form of oil-in-water emulsions (e.g. beverages, dressings, soups, sauces, desserts, yogurts), it is relatively easy to incorporate nutraceutical-loaded emulsions into them. In addition, emulsions can easily be converted into powdered form by freeze- or spray-drying, which means that encapsulated nutraceuticals can also be incorporated into dry foods, such as bakery products (Kharat *et al.,* 2017). More recently, oleogels have been also proposed as efficient tools to modulate the delivery of nutrients and bioactive molecules solubilized in the oil phase. Nevertheless, little and fragmented information can be found in the scientific literature on this topic, as the interest of the food sector on oleogelation was mostly focused on their potential application as plastic fat replacers (Patel & Dewettinck, 2016; A. Singh, Auzanneau, & Rogers, 2017; Wang, Gravelle, Blake, & Marangoni, 2016).

Oil gelation is a relatively novel strategy in which liquid oils are converted into semi-solid materials (Co & Marangoni, 2012; Patel & Dewettinck, 2016). A wide number of different oleogelators has been proposed in the literature, but they all directly gel liquid oil by self-assembly in a network after being melted in oil. Most low molecular weight structuring agents form crystalline networks entrapping and retaining oil (Co & Marangoni, 2012). The obtained oleogel structure strictly depends on crystal shape, number, and size, as well as on physical interactions among building blocks. On the other hand, phytosterol-sterol ester mixtures (e.g. β-sitosterol and γ-oryzanol) generate fibrillary networks due to their ability

to assemble in oil forming helical ribbon tubules (A. Bot & Agterof, 2006; Calligaris, Mirolo, Da Pieve, Arrighetti, & Nicoli, 2014). Finally, the polymers ethylcellulose (EC) and chitin directly produce oil gelation through the formation of a network via hydrogen bonding between polymer strands (Davidovich-Pinhas, 2016) and a particle filled network by polymer aggregation (Nikiforidis & Scholten, 2015), respectively.

Only few studies are available on the effect of oleogelation on lipolysis extent and delivery of bioactive compounds. O'Sullivan, Davidovich-Pinhas, Wright, Barbut, and Marangoni (2017) studied an oleogel containing ethylcellulose enriched with β-carotene, observing that oil structuring reduced lipolysis extent, consequently increasing the residence time of β-carotene in the digestive tract resulting in an evenly arrayed absorption over time, thus avoiding the spikes and drops in plasma concentration. A similar effect on human blood triglyceride concentration was observed by Tan, Wan-Yi, Marangoni, and Henry (2017) when comparing the co-ingestion of a carbohydrate-rich meal with EC-oleogel instead of liquid oil. Moreover, according to Yu, Shi, Liu, and Huang (2012), monostearin may increase the stability of oleogel-loaded curcuminoids by preventing their recrystallization or precipitation. However, these authors did not observe differences between oleogel and liquid oil in terms of curcuminoids bioaccessibility. Besides this evidence, the literature does not report any study on the effect of gelator type neither on lipid digestion extent nor on the fate of curcuminoids during digestion.

2.1 Effect of oleogelator type on lipolysis kinetics and curcuminoid bioaccessibility upon in vitro digestion of sunflower oil-based oleogels

2.1.1 Aim of the study

The aim of the present study was to investigate the effect of gelator type and oleogel structure on oil lipolysis during *in vitro* digestion and on the bioaccessibility of curcuminoids as loaded lipophilic bioactive compounds. The results here presented have been published:

- ✓ Calligaris, S., Alongi, M., Lucci, P., Anese, M. (2020). Effect of different oleogelators on lipolysis and curcuminoid bioaccessibility upon *in vitro* digestion of sunflower oil oleogels. *Food Chemistry*. *DOI: 10.1016/j.foodchem.2019.126146*.

2.1.2 Material and methods

2.1.2.1 Materials

High oleic sunflower oil was kindly provided by Olitalia srl (Forlì, Italy) and turmeric extract (NNCL2065, ext. dry conc. std 20:1) was purchased from Network Nutrition – IMCD spa (Milan, Italy). Bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (C) analytical standards, α-amylase from *Bacillus sp.*, porcine pepsin, porcine lipase, porcine bile extract, amyloglucosidase from *Aspergillus niger*, HCl, NaOH, $CaCl_2(H_2O)_2$, Na_2CO_3, $NaHCO_3$, NaCl, KCl, KH_2PO_4, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, $MgSO_4$ were purchased from Sigma Aldrich (Milan, Italy). Myverol™ saturated monoglycerides (fatty acid composition: 1.4% C14:0, 59.8% C16:0, 38.8% C18:0; melting point 68.05 ± 0.5 °C) were purchased from Kerry Bioscience (Bristol, UK); β-sitosterol (75.5% β-sitosterol, 12.0% β-sitostanol, 8.4% campesterol, 3.0% other) and γ-oryzanol (99% purity) were purchased from Nutraceutica srl (Monterenzio, Italy); rice wax was purchased from Kahl GmbH & Co. KG (Reinbek, Germany). All solvents were purchased from Sigma–Aldrich (Milan, Italy). Acetonitrile and 2-isopropanol were of HPLC grade. Deionized water (System advantage A10®, Millipore S.A.S, Molsheim, France) was used for all the analyses. Standard stock solutions of DMC (0.25 mg/mL), BDMC (0.25 mg/mL) and C (0.5 mg/mL) were prepared in acetonitrile.

Intermediate working curcuminoid (CUs) solutions were prepared weekly from the stock standard solution by appropriate dilution with acetonitrile and stored in the dark at 4 °C.

2.1.2.2 Oil enrichment

The turmeric extract was added to sunflower oil (5 mg/g, w/w) and the mixture was stirred for 2 h at 80 °C in the dark under nitrogen atmosphere, to avoid CUs and oil oxidation. The mixture was cooled to room temperature and filtered (Chromafil PET-20/25, 0.20 μm, 25 mm Düren, Germany) to remove extract insoluble particles.

2.1.2.3 Oleogel preparation

Oleogels were prepared by mixing CUs-enriched sunflower oil with 5% (w/w) of saturated monoglycerides (MG) (da Pieve, Calligaris, Co, Nicoli, & Marangoni, 2010), rice waxes (RW) (Doan, Van De Walle, Dewettinck, & Patel, 2015), or a mixture of β-sitosterol and γ-oryzanol (PS) (2:3 w/w) (Calligaris *et al.*, 2014). The mixtures were heated under stirring in dark conditions for 30 min at 80 °C for monoglycerides and rice wax, and for 45 min at 90 °C for phytosterols, until melting was reached. Finally, the samples were quiescently cooled to 20 °C, excluding the mixture containing γ-oryzanol and β-sitosterol that was cooled to 4 °C, stored at this temperature until the gel was formed. Samples were then analyzed after the gel setting at 20 °C.

2.1.2.4 Oleogel storage

Aliquots of 5 g of CUs-enriched oil and oleogels were placed into 10 mL vials and stored at 20 °C under dark for increasing time. Samples were collected after 60 and 100 days and analyzed for curcuminoid content.

2.1.2.5 Firmness

Oleogel firmness was determined using a texture analyzer (TA.XT Plus, Stable Micro Systems Ltd, Godalming, UK) equipped with a 5 kg load cell. A 25-mm-thick sample was compressed in between two parallel plates at a crosshead speed of 1.5 mm/s (Giacintucci *et al.*, 2018) and firmness was expressed as the maximum force (N) applied to the samples.

2.1.2.6 Rheological measurement

Rheological properties of oleogels were determined with a Haake Rheostress 6000 (Thermo Scientific, Rheostress, Haake, Germany). Aliquots of about 5 g of sample were transferred on a 40-mm parallel-plate geometry system thermostated at 20 °C and the measuring gap was set at 2 mm. Samples were left to rest for 5 min after loading before testing to allow relaxation. Stress sweep measurement in the range 0.1 to 1000 Pa was carried out at 1 Hz frequency to determine the linear viscoelastic region. Frequency sweep was carried out by applying a fixed stress value chosen in the linear viscoelastic region with a frequency scan of 0.1 to 10 Hz. Data were acquired and managed by applying the software Haake Rheowin v.4.60.0001 (Thermo Fisher Scientific The critical stress was computed as the stress leading to a 10% G' decrease during the stress sweep. G' and G" were compared at 1 Hz in the frequency sweep results. The tangent of the phase angle (tanδ) was computed as the ratio between the two moduli (G"/G') during the frequency sweep.

2.1.2.7 Macroscopic appearance

Gel images were acquired by using an image acquisition cabinet (Immagini and Computer, Bareggio, Italy) equipped with a digital camera (EOS 550D, Canon, Milano, Italy). The digital camera was placed on an adjustable stand positioned 40 cm in front of a black cardboard base where the sample was placed. Light was provided by four 23 W frosted photographic floodlights, in a position allowing minimum shadow and glare. Other camera settings were: shutter time 1/250 s, F-Number F/2,8 and focal length 60 mm. Images were saved in jpeg format resulting in pictures of 5184 × 3456 pixels, 72 × 72 dpi.

2.1.2.8 Polarized light microscopy

Polarized light microscopy was carried out by using a Leica DM 2000 optical microscope under polarized light conditions (Leica Microsystems, Heerbrugg, Switzerland). A small portion of gel was placed on a glass slide, covered with a cover slide and observed at 20 °C. The images were taken at 200× magnification using a Leica EC3 digital camera and elaborated with the Leica Suite Las EZ software (Leica Microsystems, Heerbrugg, Switzerland).

2.1.2.9 *In vitro* digestion

In vitro digestion was carried out according to the protocol proposed by Minekus *et al.* (2014). Briefly, the simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids were prepared and stored at 4 °C. The fluids were preheated to 37 °C just before *in vitro* digestion. The oral phase was started by adding to 0.25 g sample (oil or oleogel), 6 μL of $CaCl_2(H_2O)_2$ (0.3 M), 194 μL of water and 800 μL of a 6.4 mg/mL α-amylase solution, prepared in SSF and providing 75 U/mL activity in the final mixture. The sample was maintained at 37 °C under stirring for 2 min. At the end of the oral phase, the pH was adjusted to 3.0 with 40 μL HCl (1 M). Subsequently, 140 μL water and 1.82 mL of a 0.31 mg/mL pepsin solution, prepared in SGF and providing 2,000 U/mL activity in the final mixture, were added to start the gastric phase. The mix was stirred at 37 °C for up to 2 h. At the end of the gastric phase, the pH was adjusted to 7.0 with 30 μL NaOH (1 M). The intestinal phase was initiated by adding 8 μL $CaCl_2(H_2O)_2$ (0.3 M), 262 μL of water, 3.2 mL of 22.15 mg/mL lipase solution, prepared in SIF and providing 100 U/mL activity in the final mixture, and 0.5 mL of 160 mM bile extract prepared in SIF. The mix was stirred at 37 °C for up to 2 h. At the end of the intestinal phase, samples were centrifuged at 30,000 ×*g* for 70 min at 4 °C (Beckman Avanti tm J-25, Beckman Instruments Inc., Palo Alto, CA, USA), and the supernatant, i.e. the mixed micellar phase, was collected and frozen (K. Ahmed, Li, McClements, & Xiao, 2012).

2.1.2.10 Particle size and zeta potential of digested samples

The particle size distribution of digested oil and oleogel was measured by dynamic laser light scattering (Zetasizer NanoZS, Malvern Instruments, Worcestershire, UK). Samples were diluted 1:100 (v/v) with deionized water and placed in a cell where the laser light, set at 173 ° angle, was scattered by the particles. Particle size was reported as volume-weighed mean diameter in nm. The ζ-potential was also measured by placing the diluted sample in a capillary cell equipped with two electrodes to assess particle electrophoretic mobility.

2.1.2.11 Free fatty acid release and lipolysis kinetics

The amount of free fatty acids (FFA) released from the sample during the intestinal phase of *in vitro* digestion was measured by using a titration method to determine the digestion extent (K. Ahmed *et al.*, 2012). In particular, immediately after the addition of lipase, the pH of the digestion mixture was monitored and maintained at 7.0 ± 0.1 by adding 0.25 M NaOH.

The volume of NaOH added to the sample was recorded and used to calculate the percentage of free fatty acids (FFA, %) released during lipolysis (Equation 1 and Equation 2):

$$V_t = 2 \times \left[\frac{m_{oil}}{MW_{oil}} \frac{1000}{C_{NaOH}}\right] \quad \text{Equation 1}$$

$$\text{FFA (\%)} = \frac{V_e}{V_t} \times 100 \quad \text{Equation 2}$$

where V_e was the volume of NaOH used for the titration, V_t was the theoretical volume required to titrate the fatty acids released from the complete hydrolysis of the triglycerides present in the reaction vessel, assuming 2 FFA produced per 1 triacylglycerol molecule (L) (Y. Li, Hu, Du, Xiao, & McClements, 2011), m_{oil} was the mass of oil present in the reaction vessel (g), MW_{oil} was the average molecular weight of sunflower oil (g mol^{-1}) and C_{NaOH} was the concentration of the sodium hydroxide used during titration (mol L^{-1}). The maximum value of free fatty acids released during digestion was considered as an indication of the maximum lipolysis extent and was reported as FFA_{max}.

2.1.2.12 Curcuminoid quantification and bioaccessibility computation

CUs enriched oil and oleogels were diluted in isopropanol (100 μg/mL) and transferred to glass vials for UHPLC analysis, while the mixed micellar phase recovered after *in vitro* digestion was submitted to an extraction procedure. Two mL of water was added to the digested samples followed by 2 mL of isopropanol. The sample was vigorously hand-shaken for 1 min and vortexed for 15 s. A mixture of salts ($MgSO_4$/NaCl 2:1.5 w/w) was then added, shaking was repeated under the same conditions and the resulting mixture was centrifuged at 5000 ×g for 15 min to allow inorganic/organic phase separation. The supernatant was recovered and transferred to a glass vial for UHPLC analysis.

To validate the extraction procedure for CUs analysis in digested samples, accuracy was evaluated by means of recovery experiments, analyzing digested samples fortified with three different amounts of CUs (5, 10 and 20 μg for each CUs), whereas precision, expressed as the repeatability of the method, was determined in terms of relative standard deviation (RSD) from recovery experiments at each fortification level. The average recovery ranged from 92 to 100% with repeatability (CV%) lower than 2% in all cases, revealing the suitability of the procedure for the quantitative extraction of CUs from digested samples.

The chromatographic separation was performed under isocratic condition using an Agilent Poroshell C18 column (150 mm x 4.6 mm x 2.7 µm) thermostated at 30 °C and mounted on

a Shimadzu Prominence LC-20A System (Shimadzu, Milan, Italy). The mobile phase consisted of a mixture of 0.2% aqueous phosphoric acid water and acetonitrile (85:15, v/v) at 450 μL/min flow rate. The excitation (λ_{ex}) and emission (λ_{em}) wavelengths were set at 430 and 524 nm, respectively, and detection was carried out with a fluorimeter detector (Shimadzu, Milan, Italy). Calibration curves were obtained for each curcuminoid (0.1 - 500 ng on column) and presented $R^2 > 0.998$ in all cases.
CUs bioaccessibility was calculated as the percentage fraction of compound incorporated in the micelles after *in vitro* digestion *versus* its concentration in the undigested sample.

2.1.2.13 Statistical analysis

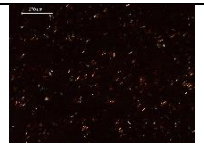
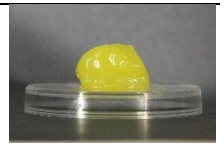
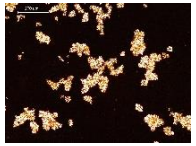
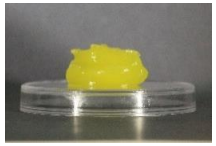
Results are averages of three measurements carried out on two replicated experiments and are reported as means ± standard deviation. Analysis of variance (ANOVA) was performed using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance and the Tukey test was used to test for differences between means ($p < 0.05$). Non-linear regression analysis of lipolysis kinetics was performed by using TableCurve2D software (Table Curve 2D 4.0, SPSS Inc., Chicago, IL, USA). The goodness of fit was evaluated based on statistical parameters of fitting (R^2, p) and the residual analysis.

2.1.3 Results and discussion

2.1.3.1 Oleogel physical and chemical properties, and curcuminoid stability

Oleogels containing sunflower oil enriched with CUs were prepared by adding 5 % (w/w) of the selected gelators. This content was previously reported to be effective in gelling vegetable oils (Calligaris *et al.*, 2014; da Pieve *et al.*, 2010; Doan *et al.*, 2015). Moreover, considering oleogelators have to be included in food formulations as additives (European Commission, 2008), this content can be considered suitable for further practical food applications.
shows firmness, rheological parameters (G', G'', Tanδ and critical stress) and microscopic and macroscopic images of oleogels obtained by using CUs enriched sunflower oil and MG, RW and PS as gelators.

Table 5. Firmness, G', G'', Tanδ, critical stress, and microscopic and macroscopic images of oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS). Different letters (a-c) mean significant differences ($p < 0.05$) of structural properties among oleogels. n.a. Images not acquired.

Gelling agent	Firmness (N)	G' (Pa)	G'' (Pa)	Tanδ	Critical stress (Pa)	Microscopic appearance	Macroscopic appearance
MG	0.91 ± 0.08 ^{c}	25090 ± 4044 ^{b}	3075 ± 513 ^{b}	0.15 ± 0.01 ^{b}	6.6 ± 0.1 ^{b}		
RW	3.49 ± 0.24 ^{b}	37413 ± 2591 ^{a}	4384 ± 539 ^{ab}	0.15 ± 0.01 ^{b}	16.7 ± 3.1 ^{b}		
PS	5.65 ± 0.42 ^{a}	29410 ± 4646 ^{ab}	6026 ± 808 ^{a}	0.21 ± 0.01 ^{a}	128.5 ± 28.6 ^{a}	n.a.	

The gel behavior was confirmed in all considered samples by rheological parameters revealing G' higher than G'' and Tanδ < 1 (Zetzl *et al.*, 2014). Considering the storage (G') and loss (G'') moduli at 1 Hz, these parameters resulted in a similar range for the three samples, even if the MG-based sample showed the lowest values. On the other hand, PS demonstrated the highest critical stress value, which represents the beginning of the non-linear region, accounting for the structure breakdown required to onset flowing (Doan *et al.*, 2015). This result is consistent with firmness data (PS >RW>MG) and can be associated with the peculiar arrangement of β-sitosterol/γ-oryzanol mixture. As well reported by (Sawalha *et al.*, 2015), these molecules self-assemble into a network made of cross-linked tubules aligned as helical ribbon stabilized by hydrogen bonds. The network structure formed by β-sitosterol/γ-oryzanol mixture was able to confer to the system a higher resistance to stresses in comparison to the network formed by MG and RS. It should be remembered that the ability of MG and RW to gel oil is associated to their crystallization behavior and the resulting network is based on interactions among crystalline aggregates (da Pieve *et al.*, 2010; Doan *et al.*, 2015) well visible in the polarized light microscopy images (Table 1). It can be noted that small needle-like crystals were present in MG containing system; whereas larger spherical crystals were observed in RW containing sample, in agreement with the literature (da Pieve *et al.*, 2010; Doan *et al.*, 2015). These differences in crystal morphology led to the different firmness of the samples. As expected, the tubes made of β-sitosterol/γ-oryzanol mixture cannot be imaged with this technique being these structures of dimensions around 10 nm, smaller than the wavelength of visible light and thus resulting in transparent systems (A. Bot & Agterof, 2006)(A. Bot, Den Adel, & Roijers, 2008). The β-sitosterol/γ-oryzanol tubular structure in oil was previously imaged by SEM by (Sawalha *et al.*, 2015).

As changes in the physical properties were observed when different gelators were used, further research was carried out to investigate CUs content after gel preparation. As already mentioned, oleogels were prepared by heating oil at temperatures higher than the melting temperature of the considered gelling molecules. Being CUs sensitive to heat, their degradation could occur during oleogel preparation. The concentration of BDMC, DMC, and C in the turmeric extract, in the enriched oil and in the oleogels was thus determined by UHPLC. BDMC, DMC, and C concentration in turmeric extract accounted for 10,142 ± 10 µg/g, 53 277 ± 79 µg/g and 147,761 ± 249 µg/g, respectively. As expected, C was the most abundant compound, followed by BDMC and DMC (Yu & Huang, 2012). Based on CUs concentration in the turmeric extract and in freshly prepared oil (Table 6), it can be observed that these compounds were completely solubilized during oil enrichment. BDMC,

DMC and C concentration in oleogels did not differ significantly from those found in freshly prepared oil and ranged between 39 and 41, 264 and 274, and 1,049 and 1,112 $\mu g/g_{oil}$, respectively. These results suggest that the preparation methodology applied to produce oleogels did not induce CUs degradation. It can be noted that BDMC, DMC, and C accounted for 2%, 14% and 84% of CUs, respectively (Table 6).

Table 6. Bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (C) contents in oil and oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW), and phytosterols (PS), during storage at 20 °C under dark. Different capital letters (A-C) mean significant differences of curcuminoid concentration during storage. Different lowercase letters (a-c) mean significant differences ($p < 0.05$) of curcuminoid concentration between oil and oleogels.

	BDMC ($\mu g/g_{oil}$)			DMC ($\mu g/g_{oil}$)			C ($\mu g/g_{oil}$)		
Sample	0 days	60 days	100 days	0 days	60 days	100 days	0 days	60 days	100 days
Oil	$41.2 \pm 1.8^{A,a}$	$38.5 \pm 0.2^{A,a}$	$31.6 \pm 0.1^{B,a}$	$274 \pm 12^{A,a}$	$251 \pm 1^{A,a}$	$193 \pm 3^{B,b}$	$1112 \pm 48^{A,a}$	$974 \pm 5^{B,a}$	$820 \pm 5^{C,b}$
MG	$39 \pm 1.5^{A,a}$	$38.9 \pm 1.8^{A,a}$	$32.1 \pm 0.3^{A,a}$	$268 \pm 10^{A,a}$	$250 \pm 12^{A,a}$	$192 \pm 2^{A,b}$	$1049 \pm 38^{A,a}$	$974 \pm 49^{A,a}$	$812 \pm 6^{B,bc}$
RW	$41.0 \pm 2.8^{A,a}$	$37.3 \pm 0.3^{A,a}$	$29.8 \pm 0.2^{B,b}$	$272 \pm 15^{A,a}$	$246 \pm 1^{A,a}$	$200 \pm 2^{B,ab}$	$1104 \pm 57^{A,a}$	$968 \pm 2^{A,a}$	$794 \pm 3^{B,c}$
PS	$39.7 \pm 2.3^{A,a}$	$38.2 \pm 0.3^{A,a}$	$31.3 \pm 0.2^{B,a}$	$265 \pm 15^{A,a}$	$247 \pm 2^{A,a}$	$207 \pm 1^{B,a}$	$1079 \pm 58^{A,a}$	$1007 \pm 8^{A,a}$	$853 \pm 3^{B,a}$

Samples were further analyzed for CUs content during storage at 20 °C. As shown in , CUs concentration did not change up to 60 days of storage, whereas a significant decrease was recorded at 100 days of storage in all samples. In any case, the measured differences did not allow to highlight a clear effect of different structuring agents on CUs stability.

2.1.3.2 Physical properties, lipolysis kinetics and bioaccessibility of *in vitro* digested oleogels

In the second part of the research, the particle size distribution of oil and oleogels was investigated after *in vitro* digestion (Figure 6a) to understand whether the use of different structuring agents affected the characteristics of the digested matrices.

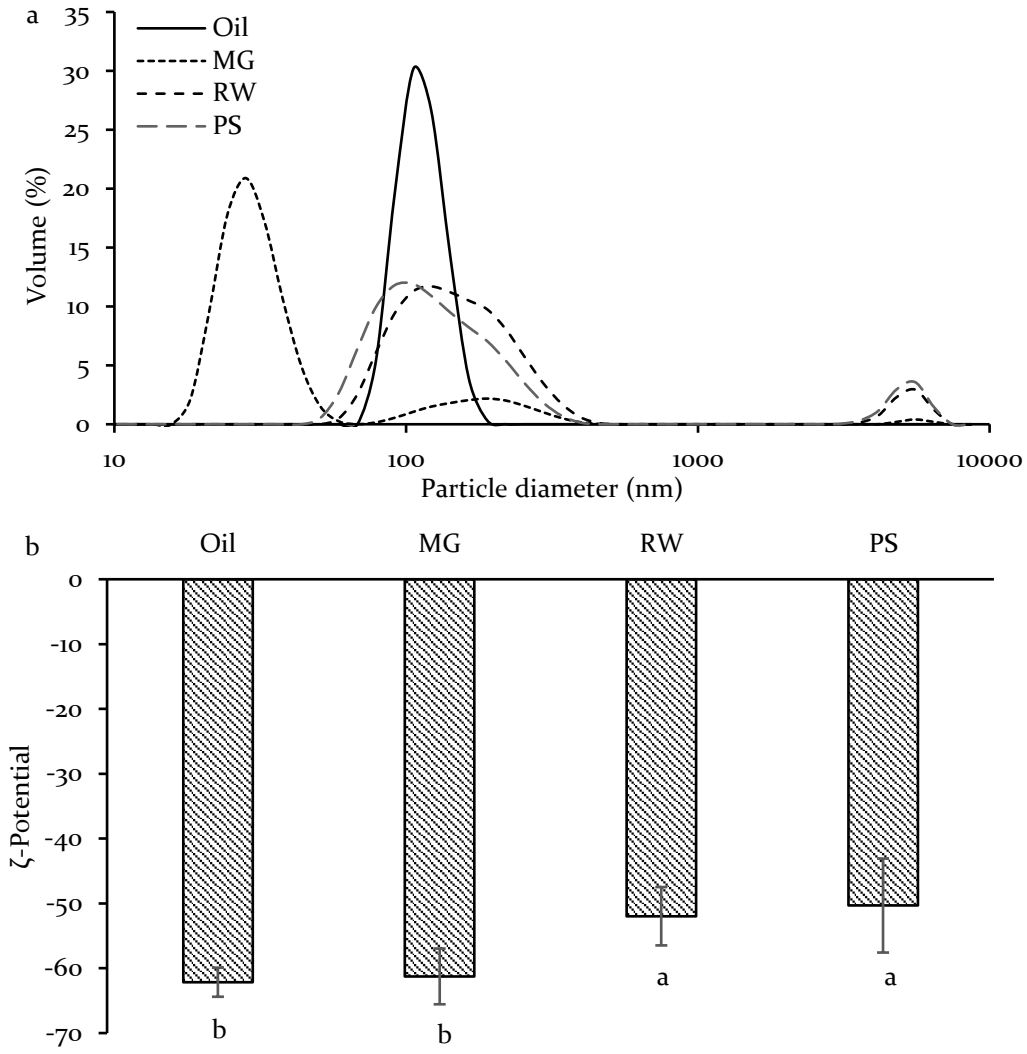


Figure 6. Particle size distribution (a) and ζ-potential (b) of oil and oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS), after *in vitro* digestion. Different letters (a-c) mean significant differences ($p < 0.05$) among samples.

A multimodal particle size distribution was observed for all samples, revealing the presence of smaller and larger particles. In most cases, the major family showed an average diameter ranging from 90 to 190 nm, likely attributable to mixed micelles formed upon digestion (Salvia-Trujillo *et al.*, 2017). Differently, MG major family presented an average particle diameter of 28 nm, while the peak corresponding to 190 nm accounted for a much lower volume as respect to the other oleogels. Such a difference could be attributed to the ability of MG to act as surfactants during *in vitro* digestion, thus promoting the formation of smaller micelles (Reis *et al.*, 2008). On the contrary, the RW, EC and PS gelators showed a broader particle size distribution with a larger average diameter, suggesting possible interference with the digestion process (O'Sullivan *et al.*, 2017). In fact, the lipid-surrounding matrix can create a physical barrier able to prevent the access of lipase to the lipid or can directly interfere with lipid digestive components (McClements, Decker, & Park, 2009), resulting in a less efficient micelle formation. In this regard, it is important to highlight that micellarization plays a pivotal role in determining the absorption of loaded lipophilic bioactive compounds, but no correlation has been highlighted between the size and the bioaccessibility (Salvia-Trujillo *et al.*, 2017). As shown in Figure 6a, all analyzed samples also presented a particle family with an average diameter of 4,800-5,500 nm that can be attributed to the presence of undigested lipid droplets and anionic species, such as free fatty acids and bile salts (Salvia-Trujillo, Qian, Martín-belloso, & McClements, 2013; H. Singh, Ye, & Horne, 2009; Zou, Zheng, Zhang, Zhang, & Liu, 2016). The latter may form complex colloidal structures presenting a highly negative charge (R. Zhang *et al.*, 2016). In this regard, Figure 6b shows the ζ-potential of digested oil and oleogels after *in vitro* digestion. This parameter provides information about the surface electrical charge of particles in the mixed micellar phase and depends on the adsorbed species at the oil-water interfaces (Salvia-Trujillo *et al.*, 2017). All digested oleogels presented negatively charged particles, with a ζ-potential ranging from -50 to -62 mV. In fact, the micelles formed during digestion are characterized by the presence of complex colloidal structures formed from bile salts and phospholipids or anionic free fatty acids deriving from lipid hydrolysis and contributing to the overall negative charge of micelles (H. Singh *et al.*, 2009). Unstructured oil and MG-containing oleogel were characterized by the most negatively charged micelles upon digestion, whereas RW- and PS-containing samples presented fewer negative charges, confirming the effect of different structuring agents on the formation of micelles during digestion, as observed in the case of the size distribution (Fig. 1a).

Moreover, some authors observed a relationship between the electrical charge upon digestion and the lipolysis degree (Qian, Decker, Xiao, & McClements, 2012). Lipid digestion was thus monitored by measuring free fatty acid (FFA) release during the intestinal phase of *in vitro* digestion (Figure 7).

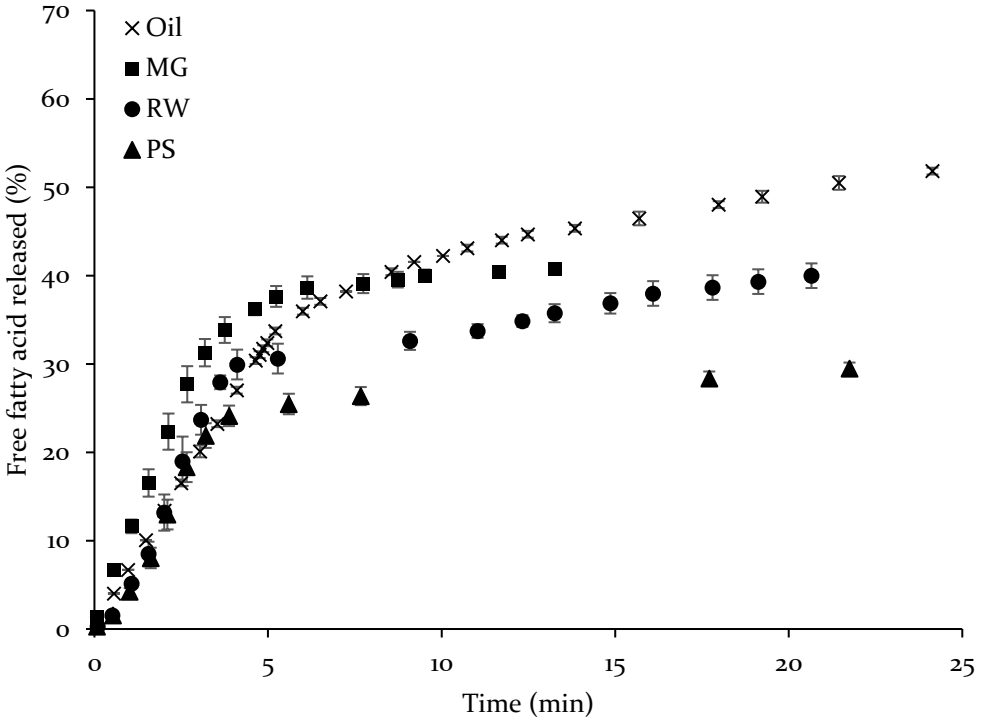


Figure 7. Concentration of free fatty acids (FFA) during *in vitro* digestion in oil and oleogels containing 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS).

In can be observed that the FFA release increased during digestion for all analyzed samples and reached a *plateau* within 30 min, in agreement with literature data (McClements & Li, 2010; O'Sullivan *et al.*, 2016).

However, the extent of lipid digestion was significantly affected by oil structure. The unstructured oil presented the maximum extent of lipid digestion (FFA_{max} = 52%) in comparison with oleogels. This value agreed with literature findings, reporting a maximum lipolysis extent for unstructured sunflower oil around 57% (Ye *et al.*, 2019). Among oleogels, MG and RW presented a similar lipolysis extent (nearly 40%), whereas PS-based oleogel showed the lowest lipolysis degree (less than 30%) upon *in vitro* digestion, being 20% lower than that of oil. These results suggest that the presence of differently structured supramolecular networks in oil affected the kinetics of lipid digestion probably by hindering the lipase access to triacylglycerol digestion sites. Thus, the final strength of the oleogel instead of the molecular structure of the oleogelator seems to be the most critical factor

affecting lipolysis, in agreement with (Ashkar, Laufer, Rosen-Kligvasser, Lesmes, & Davidovich-Pinhas, 2019) studying canola oil-based oleogels containing MG and PS. It should be noted that no universal conclusion on the relation between the gel strength and lipolysis can be gathered, as both gel strength (Sawalha *et al.*, 2015; S. Yang *et al.*, 2018) and lipolysis extent (Ye *et al.*, 2019) may be differently affected by fat composition. Still, it can be inferred that it may be possible to modulate digestibility and fat availability by selecting a proper oleogel structure.

Several authors (Qian *et al.*, 2012; Salvia-Trujillo *et al.*, 2017) observed an interdependence between lipolysis efficiency and micelle characteristics. In fact, besides resulting in the highest lipolysis extent (Figure 7), MG-containing oleogel also presented the smallest and most negatively charged mixed micelles (Figure 6), comparable with those observed in the unstructured oil. Since these differences could affect the bioaccessibility of loaded lipophilic bioactive compounds, to get an insight into the effect of different structuring agents on this feature, CUs concentration and bioaccessibility in the *in vitro* digested enriched oil and oleogels were assessed, and results are shown in Table 7.

Table 7. Bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC) and curcumin (C) content and bioaccessibility (BAC) in oil and oleogels obtained by using 5% (w/w) of monoglycerides (MG), rice wax (RW) and phytosterols (PS) after *in vitro* digestion. Different letters (a-c) mean significant differences ($p < 0.05$) of curcuminoid concentration or bioaccessibility among samples.

Sample	BDMC		DMC		C	
	Concentration ($\mu g/g_{oil}$)	BAC (%)	Concentration ($\mu g/g_{oil}$)	BAC (%)	Concentration ($\mu g/g_{oil}$)	BAC (%)
Oil	22.9 ± 3.8 ^{a}	55.6 ± 9.1 ^{a}	141.9 ± 22.8 ^{a}	51.7 ± 8.3 ^{a}	520.4 ± 85.9 ^{a}	50.4 ± 7.7 ^{a}
MG	13.1 ± 0.5 ^{c}	33.6 ± 1.4 ^{b}	87.3 ± 5.5 ^{b}	32.5 ± 2.1 ^{c}	333.9 ± 31.5 ^{b}	31.8 ± 3.0 ^{b}
RW	13.7 ± 3.4 ^{bc}	33.3 ± 8.4 ^{b}	94.0 ± 22.6 ^{b}	34.5 ± 8.3 ^{bc}	376.5 ± 93.0 ^{ab}	29.2 ± 8.4 ^{b}
PS	20.5 ± 2.0 ^{ab}	51.4 ± 5.0 ^{a}	130.5 ± 11.1 ^{ab}	49.1 ± 4.2 ^{ab}	489.3 ± 39.7 ^{ab}	45.2 ± 3.7 ^{a}

As well known, BAC measures the percent transfer of the bioactive molecules from the lipid phase to the aqueous phase, in which CUs are incorporated into mixed micelles (Ferruzzi, 2010). Only the CUs that are present in the micellar phase are then available to be absorbed by the cells of the intestinal epithelium. A significant decrease ($p<0.05$) in the concentration of CUs was observed for both oil and oleogels in the micellar phase after *in vitro* digestion, as compared to undigested samples (Table 6). Regarding the susceptibility of different CUs

upon digestion, curcumin presented the lowest BAC, being more susceptible to oxidation as compared to BDMC and DMC (Gordon, Luis, Ashley, Osheroff, & Schneider, 2015), in agreement with the results reported by Yu *et al.* (2012). In addition, the structuring agent significantly affected CUs concentration and bioaccessibility in digested samples, in contrast with the results found for the undigested oleogels (Table 6). In particular, oleogels made of crystalline gel network (MG and RW-based oleogels) presented the lowest CUs content and bioaccessibility upon *in vitro* digestion. It can be speculated an effect of the presence of crystalline particles on CUs BAC. In particular, CUs could be involved in the gelation/crystallization process being more exposed to oxygen and thus more prone to faster oxidation. Similar hypotheses have been made on β-carotene oxidative degradation in structured lipids by different authors (Calligaris, Valoppi, Barba, Anese, & Nicoli, 2018; A. J. Martins, Cerqueira, Cunha, & Vicente, 2017).

By contrast, the bioaccessibility of CUs in PS oleogel was comparable to that of oil, suggesting it was not affected by oil structure. Differently from lipolysis, these results seem to highlight an effect of gel network type rather than of gel strength on CUs bioaccessibility. It can be speculated that the possible interactions between CUs and gelator network structure could be crucial in determining the oxidative stability of CUs during simulated digestion.

2.1.4 Conclusions

Results reported in the present study confirmed that oleogelation could be a profitable strategy to modulate lipid digestion while delivering bioactive molecules. The oleogel structure seems to affect lipolysis kinetics and the bioaccessibility of loaded lipophilic bioactive compounds concomitantly. Regarding lipid digestion, the gel strength was the critical factor affecting the rate and extent of lipolysis. On the contrary, the choice of the gelling agent had an impact on CUs bioaccessibility.

Based on this knowledge, it can be suggested that oleogel development would not only offer to the food industry a plastic fat replacer but could represent a strategy to modulate lipid digestion and deliver health benefits. Matching the reduction of fat uptake and the improved bioaccessibility of bioactive molecules would provide the consumer with a functional food potentially able to tackle the risk of obesity, cardiovascular diseases and diabetes, currently representing the major diseases concerning public health.

Chapter 3 Coffee

Coffee was discovered in Ethiopia in the fifteenth century and since then its consumption has rapidly spread first in Europe and later worldwide (Nehlig, 1999). Currently coffee represents one of the most marketed food items (Esquivel & Jiménez, 2012).

Consumers mainly drink it as a stimulant, due to its caffeine content, which can increase the alertness, the energy, and the ability to concentrate. Nonetheless several health benefits have also been attributed to coffee (Ludwig, Clifford, *et al.*, 2014), including the protection against Parkinson (G. Hu, Bidel, Jousilahti, Antikainen, & Tuomilehto, 2007), Alzheimer (Lindsay *et al.*, 2002) and cardiovascular diseases (Kleemola, Jousilahti, Pietinen, Vartiainen, & Tuomilehto, 2000), as well as antihypertensive, anti-inflammatory, immunoprotective, anti-cancer (Palmioli *et al.*, 2017), anti-aging (Amigoni *et al.*, 2017), neuroprotective (Ciaramelli, Palmioli, & Airoldi, 2019) and antidiabetic (Iwai *et al.*, 2012; Johnston, Clifford, & Morgan, 2003) effects.

In particular, several epidemiological studies showed that a moderate and prolonged coffee consumption contributes to the reduction of type 2 diabetes risk (Lecoultre *et al.*, 2014; van Dam & Hu, 2005; Van Dijk *et al.*, 2009), up to 60%, being this value is in the same range as observed with pharmacological approaches (Ludwig, Clifford, *et al.*, 2014).

This effect was associated with the ability of some compounds contained in coffee to reduce the blood glucose level, thus promoting a hypoglycemic effect (Johnston *et al.*, 2003; Krebs, Parry-strong, Weatherall, Carroll, & Downie, 2012; Salazar-Martinez *et al.*, 2004; Shearer *et al.*, 2003). This is mostly attributed to coffee phenolic composition (Gökcen & Şanlier, 2019) and in particular to chlorogenic acids (quinic esters of hydroxycinnamic acids, CGA), which are the most important class of coffee polyphenols (12–18% dry weight in green coffee). Their mechanism of action is still not completely clear (van Dam & Hu, 2005), but the going assumption is the attenuation of carbohydrate digestion through α-glucosidase inhibition (Hanhineva *et al.*, 2010; Williamson, 2013).

The α-glucosidase inhibition capacity was actually demonstrated not only for phenolic compounds, as in the case of chlorogenic acids (Iwai *et al.*, 2012; Johnston *et al.*, 2003;

Lecoultre *et al.*, 2014), caffeic acid, quercetin (Murase *et al.*, 2012) and trigonelline (Hamden, Bengara, Amri, & Elfeki, 2013; Van Dijk *et al.*, 2009), but also for Amadori compounds (Ha *et al.*, 2011) and Maillard reaction products (Hwang *et al.*, 2011). Still most studies deal with model systems, as in the case of Kim (2015) who demonstrated that a β-carboline alkaloid norharman isolated from coffee inhibits α-glucosidase with an uncompetitive mechanism.

Even though some authors (Nyambe-Silavwe & Williamson, 2018) expressed doubts about the ability of chlorogenic or phenolic acids to inhibit carbohydrate-digesting enzymes alone, still only few papers deal with the whole coffee beverage (Moreira, Nunes, Domingues, & Coimbra, 2012) and thus little is known about the overall inhibitory effect it carries out (K. Singh *et al.*, 2014). In fact, coffee consists of a complex mix of compounds, whose interaction may modify the overall effect towards α-glucosidase (Farah, De Paulis, Trugo, & Martin, 2005).

Besides the complexity of food matrix, also the effect of processing, and specifically of roasting, on the antidiabetic potential of coffee has been almost completely neglected so far. However, most of the compounds contained in the brew are produced during roasting, upon chemical modification of green coffee composition (Hečimović, Belščak-Cvitanović, Horžić, & Komes, 2011; Moreira *et al.*, 2012). Roasted coffee composition depends on roasting time and temperature, whose choice allows obtaining coffees with different degree of roast. Besides contributing to coffee flavour, roasting induces the formation of many bioactive compounds. For instance, the melanoidins developed upon Maillard reaction demonstrated antioxidant, antimicrobial and anti-inflammatory properties (F. J. Morales, Somoza, & Fogliano, 2012).

Thus, by substantially modifying coffee composition, different roasting conditions may potentially affect its α-glucosidase inhibitory capacity.

As in the case of the degrees of roast, which intensity is differently appreciated around the world, there are also significant differences in the consumption patterns, including brew preparation and the addition of ingredients, such as sugar and milk.

The consumption of ready-to-drink milk beverages supplemented with coffee has actually increased markedly. Milk based coffee beverages can be regarded as emulsions in which the lipid phase, i.e. milk fat, is dispersed in a water phase containing both coffee constituents (i.e. melanoidins, phenolic compounds, and flavors) and milk components (mainly whey proteins and caseins) (Nehlig, 1999).

Due to the presence of different phases potentially leading to physical instability, these emulsions are commonly stabilized by the application of high-pressure homogenization (HPH) (Paquin, 1999).

Besides contributing to the physical stabilization of the beverage, the formation of micelles including coffee phenolic compounds can contribute also to their protection from degradation during digestion (Otemuyiwa, Williams, & Adewusi, 2017). However, opposite findings about the role of milk addition towards bioactive compounds have been reported. In fact, other authors observed an impaired bioaccessibility of phenolic compounds upon milk addition to coffee, probably due to their binding to milk proteins (Duarte & Farah, 2011; Dupas *et al.*, 2006).

Moreover, although some studies reported on the bioaccessibility of coffee phenolic compounds considering the whole matrix and its mix with other ingredients (Tagliazucchi, Helal, Verzelloni, & Conte, 2012), no data are available on the ability of coffee to exert its antidiabetic properties, and more specifically to inhibit α-glucosidase upon the digestion process.

These considerations revealed the need to get an insight into the effect of the whole coffee matrix, the addition of ingredients and the digestion process on coffee antidiabetic properties.

3.1 Effect of coffee roasting on *in vitro* α-glucosidase activity: inhibition and mechanism of action

3.1.1 Aim of the study

The aim of this study was to investigate the effect of different roasting degrees on the *in vitro* inhibitory capacity against α-glucosidase exhibited by coffee extracts and their fractions. The mechanism of inhibition carried out by the extracts and fractions was also studied. The results here presented have been published:

- ✓ Alongi, M., Anese, M. (2018). Effect of coffee roasting on *in vitro* α-glucosidase activity: inhibition and mechanism of action. *Food Research International, 111*, 480-487.

3.1.2 Material and methods

3.1.2.1 Sample preparation

Coffee samples were prepared by using green coffee beans from the species *Coffea canephora var. robusta* from Vietnam. Coffee was roasted in an air circulation oven (TC 40 Thermocenter, Salvis, Reussbühl, Switzerland) at 200 °C during 15, 45 and 60 min. After the treatments, samples were immediately removed from the oven, cooled to room temperature and left to stand at room temperature for 12 h. The roasted samples were ground in a mill (Moulinex mod. 505, Paris, France) and sieved through a 35 Mesh sieve. Afterwards, coffee powders were transferred into plastic vessels with pressure lid and stored at -18 °C until analyses were performed. Coffee brews were prepared by solid-liquid extraction with milli-Q deionized water of the ground green and roasted beans. The ratio between coffee powder and water was 1:8 (w/w). The coffee-water mixture was boiled for 5 minutes under stirring at 300 rpm (Yellow line magnetic IKA, Staufen, Germany), cooled, filtered through filter paper and freeze-dried (Laboratory and Pilot Freeze Dryer Mini-fast, mod. 1700, Edwards Alto Vuoto, Milano, Italy). Freeze-dried material was added with a mixture of methanol and water (1:9 v/v) to obtain 0.4 g/mL coffee extracts which were used for further analyses.

3.1.2.2 Coffee fractions separation

The method of Borrelli, Visconti, Mennella, Anese, and Fogliano (2002) was followed. A HPLC system (LC-10AT VP, Shimadzu Corporation, Kyoto, Japan) equipped with a UV/VIS

detector (SPD-10 AT VP, Shimadzu Corporation, Kyoto, Japan) was used. The coffee extract was filtered (Whatman 0.45 μm) and loaded onto a Sephadex G-25 gel filtration chromatography column (60 × 1.1 cm i.d.; Pharmacia, Uppsala, Sweden). The injection volume was 20 μL and the mobile phase, delivered at a flow rate of 1.2 mL/min, was milli-Q water. The detection wavelength was 280 nm. Peak integration was performed by Polyview 2000 software (Ver. 5.3, Varian, Texas, USA). In according with (Borrelli *et al.*, 2002) four fractions were collected: fraction I, containing high-molecular-weight (HMW) material, indicated as coffee melanoidins; fractions II and III, containing intermediate-molecular-weight (IMW) compounds; and fractions IV, containing small compounds such as low-molecular-weight (LMW) phenols. Since fractions II and III both contained IMW compounds, in the present experiment they were mixed together. Thus, three fractions, i.e. high, intermediate and low molecular weight, were obtained from each coffee sample. The coffee fractions were freeze-dried and stored in a desiccator, until analyses were performed. Prior to analyses, freeze dried fractions were added with a mixture of methanol and water (1:9 v/v) to obtain coffee fractions with a concentration of 0.1 g/mL.

3.1.2.3 Weight loss and total solid content determinations

Sample weight loss upon roasting was calculated as the percentage weight difference between the initial and final weights of the coffee beans. Total solid content was determined by gravimetric method (AOAC, 1995).

3.1.2.4 Colour analysis

Color analysis was carried out on ground coffee using a tristimulus colorimeter (Chromameter-2 Reflectance, Minolta, Osaka, Japan) equipped with a CR-300 measuring head. The instrument was standardized against a white tile before measurement. Color was expressed in CIE units as L* (lightness/darkness), a* (redness/greenness) and b* (yellowness/blueness). The parameters a* and b* were used to compute the hue angle ($\tan^{-1}$ b*/a*) (F. M. Clydesdale, 1978).

3.1.2.5 Phenolic compound quantification

Chromatographic quantification of chlorogenic acids was performed on coffee extracts and fractions following the method proposed by Hečimović *et al.* (2011) modified according to Llorach, Tomás-Barberán, and Ferreres (2004). A HPLC pump (LC-10AT VP, Shimadzu

Corporation, Kyoto, Japan) equipped with a diode array detector (SPD-10 AT VP, Shimadzu Corporation, Kyoto, Japan) and an inverse phase apolar C18 column (5 µm, 250 x 4.6 mm, Alltima, Lokeren, Belgium) were used. The injection valve (Rheodyne, Sigma-Aldrich, Milano, Italy) was equipped with a 20 μL plastic loop and samples were injected using a syringe (SGE LC, 100 µL, FN). The elution was in gradient mode using a mixture of 5% formic acid (Sigma-Aldrich, Milano, Italy) in water (solvent A) and methanol (Sigma-Aldrich, Milano, Italy) (solvent B) as mobile phase at a flow rate of 1 mL/min. Gradient was set as follows: solvent A was held at 90% for the first 25 min, decreased to 80% and held at this level for 15 min; then decreased further to 50% and held for 5 min; finally, 90% solvent B was reached and held for 15 min. The detection was conducted at 280, 335 and 350 nm. Quantification was carried out using 3-caffeoylquinic (3-CQA) and 5-caffeoylquinic (5-CQA) acids as external standards. Calibration curves were linear (R^2 > 0.995) in the 2.0 to 200.0 mg/L concentration interval. Peaks integration was performed by using Polyview 2000 software (Ver. 5.3, Varian, Texas, USA). Total phenolics (TP; i.e., the overall number of phenolic compounds) was computed by summing up the concentration of phenolic compounds detected at 280, 335 and 350 nm. TP, chlorogenic acids (CGA), 3-CQA and 5-CQA concentrations were expressed as mg/g_{dm} of the coffee extract.

3.1.2.6 α-Glucosidase inhibition assay

The inhibitory activity of coffee extracts and their fractions against α-glucosidase was assessed spectrophotometrically (UV-2501PC, UV-VIS Recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan), following the method of K. Singh *et al.* (2014) with some modifications. Freeze-dried coffee extracts and fractions were diluted to proper concentrations with a mixture of methanol and deionized water (1:9 v/v). Different aliquots of coffee extracts (0.4 g/mL) and fractions (0.1 g/mL), or 3.33 mg/mL 3-CQA and 5-CQA (Sigma-Aldrich, Milano, Italy) and 0.004 mg/mL acarbose (Sigma-Aldrich, Milano, Italy) aqueous solutions were introduced in 1 mL capacity cuvettes in the presence of 30 μL α-glucosidase solution (0.04 mg/mL in 0.1 M phosphate buffer, pH=7, corresponding to 1 U/mL), and phosphate buffer (100 mM, pH 7) to the volume of 900 μL, and mixed well. After incubation at 37 °C for 10 minutes, the reaction was started by adding 100 μL of 5 mM 4-nitrophenyl-α-D-glucopyranoside (Sigma-Aldrich, Milano, Italy) solution in 100 mM phosphate buffer (pH 7.0) as substrate. Absorbance was recorded at 405 nm during 15 minutes after every 30 s. Further assays were performed using 10 µL coffee solution and varying the volume of substrate solution between 0 and 200 µL. Controls lacking inhibitors

were run and defined the control activity in each experiment. Blanks lacking the enzyme were also tested to prevent the interference of coffee extracts and fractions with color development upon 4-nitrophenyl-α-D-glucopyranoside hydrolysis. The α-glucosidase inhibition carried out by coffee extracts and fractions was calculated using Equation 3:

$$Percentage\ of\ inhibition\ = 100 - \left(\frac{k_s}{k_c} \times 100\right) \qquad \text{Equation 3}$$

where k_s and k_c were the kinetic constants in the presence and in the absence of the inhibitor, respectively. The half-maximal inhibitory concentrations (IC_{50}), i.e. the concentration of inhibitor required to produce a 50% inhibition against α-glucosidase, of coffee extracts, coffee fractions, 3-CQA, 5-CQA and acarbose were assessed by the linear regression of the inhibition percentage *versus* the inhibitor concentration. The acarbose equivalent was calculated as $IC_{50\ \text{acarbose}}/IC_{50\ \text{sample}}$ (Nasu *et al.*, 2005).

3.1.2.7 Determination of kinetic parameters of α-glucosidase in the presence and absence of coffee extracts and fractions, and identification of inhibition type

The k_m and V_{max} and apparent k_m (k_m*) and V_{max} (V_{max}*) values, respectively in absence and presence of coffee extracts and fractions (hereafter called inhibitors, I), as well as the inhibition type were determined from double reciprocal plots (Lineweaver-Burk). The inhibition constants (k_i and k_i') values were computed using Equation 4 for competitive and Equation 5 and Equation 6 for mixed-type inhibition, respectively (E. M. Ahmed *et al.*, 2014).

$$k_i = \frac{k_m(I)}{k_{m*} - k_m} \qquad \text{Equation 4}$$

$$k_i = \frac{V_{max} \times k_m \times (I)}{V_{max} k_{m*} - V_{max*} k_m} \qquad \text{Equation 5}$$

$$k_i' = \frac{V_{max}(I)}{V_{max} - V_{max*}} \qquad \text{Equation 6}$$

3.1.2.8 Statistical analysis

Data were reported as mean ± standard deviation of at least three measurements on two replicated samples. Analysis of variance (ANOVA) was performed with significance level set

to $p < 0.05$; the Bartlett procedure was used to test homogeneity of variances, using R software, version 3.4.3 (The R Foundation for Statistical Computing, 2018). Linear regression analysis was performed by using Microsoft Excel 2016. The goodness of fitting was evaluated based on visual inspection of residual plots and by the calculation of R^2 and p.

3.1.3 Results and discussion

3.1.3.1 Effect of roasting on the *in vitro* inhibitory activity of coffee extracts against α-glucosidase

Coffees with different degrees of roast were produced by submitting Robusta coffee beans to roasting for increasing lengths of time. Weight loss total solid content and color parameters (i.e. L* and hue angle) changed during roasting (Table 8), in agreement with the literature (Moreira *et al.*, 2012; Opitz *et al.*, 2014; Vargas-Elías, Correa, De Souza, Baptestini, & De C. Melo, 2016). Based on weight loss, roasted coffees were classified as medium, dark and very dark (Clarke, 1987).

Table 8. Weight loss, total solid content and color of ground Robusta coffee beans with different degree of roast, and total phenolic (TP), chlorogenic acid (CGA), 3-caffeoylquinic acid (3-CQA) and 5-caffeoylquinic acid (5-CQA) contents of the corresponding coffee extracts. Means in the same column indicated by a common letter (a-d) are not significantly different ($p > 0.05$).

Degree of roast	Ground coffee		Coffee extract					
	Weight loss (%)	Total solid content (%)	L*	Hue angle (tan^{-1} b*/a*)	TP (mg/g_{dm})	CGA (mg/g_{dm})	3-CQA (mg/g_{dm})	5-CQA (mg/g_{dm})
Unroasted	-	95.5 ± 0.1^{b}	67 ± 1^{a}	80 ± 1^{a}	16.2 ± 0.2^{a}	13.8 ± 0.6^{a}	6.4 ± 0.3^{a}	1.2 ± 0.0^{a}
Medium	9	99.4 ± 0.1^{a}	48 ± 1^{b}	68 ± 0^{b}	15.9 ± 0.1^{a}	10.8 ± 0.3^{b}	3.8 ± 0.1^{b}	1.6 ± 0.0^{a}
Dark	12	99.5 ± 0.2^{a}	37 ± 1^{c}	25 ± 6^{d}	11.3 ± 0.2^{b}	2.6 ± 0.3^{c}	0.6 ± 0.1^{c}	0.5 ± 0.0^{b}
Very dark	15	99.9 ± 0.1^{a}	25 ± 1^{d}	33 ± 1^{c}	8.4 ± 0.1^{c}	0.9 ± 0.1^{d}	0.2 ± 0.0^{d}	0.2 ± 0.0^{c}

Unroasted, medium, dark, and very dark roasted coffee extracts were analyzed for *in vitro* α-glucosidase inhibitory activity (Figure 8).

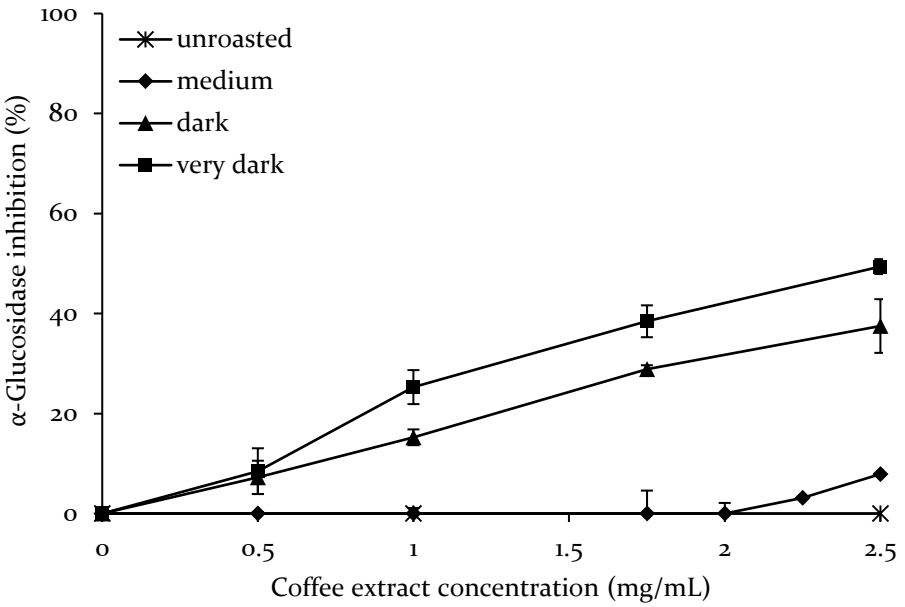


Figure 8. *In vitro* inhibitory activity against α-glucosidase of unroasted, medium, dark and very dark roasted coffee extracts, as a function of their concentration.

Coffee extracts differently affected α-glucosidase activity. In particular, the green coffee extract did not exert any inhibitory effect in the whole range of analyzed concentrations (up to 10 mg/mL). Analogously, the medium roasted coffee extract did not show any inhibitory effect at concentrations lower than 2 mg/mL, while a 7% inhibition was recorded at 2.5 mg/mL concentration. Higher coffee extract concentrations were not considered, since absorbance signals were out of the linear range. The inhibitory effect of dark and very dark roasted coffee extracts increased almost linearly with concentration, and was higher for the most intensively roasted sample, reaching a 49% value at 2.5 mg/mL concentration.

Since most functional properties of coffee have been attributed to phenolic content (Iwai *et al.*, 2012; Johnston *et al.*, 2003; Lecoultre *et al.*, 2014), TP, CGA, 3-CQA and 5-CQA were quantified in coffee extracts (Table 8), to understand whether changes in the inhibitory activity could be attributed to modifications occurring during roasting in the phenolic composition. TP content of the coffee extract obtained from unroasted beans fell within the range reported in literature, i.e. 0.2 to 40 mg/g. Such a variability is attributable not only to the species and the variety, but also to the processing conditions of coffee beans (Hečimović *et al.*, 2011; Ludwig, Mena, *et al.*, 2014). To this regard, it should be noticed that, in agreement with literature findings (M.N. Clifford, 1985; Vignoli, Bassoli, & Benassi, 2011), TP

significantly decreased upon roasting, which produced a nearly 2-fold reduction from the unroasted to the very dark roasted sample. As reported in the literature (M.N. Clifford, 1985; Vignoli *et al.*, 2011), CGA represented the major class of phenolic compounds in coffee, accounting for 85% of the overall TP content in the green coffee extract (Table 8). Upon very dark roasting, CGA were reduced by more than 90%, due to their high susceptibility to thermal degradation (Fujioka & Shibamoto, 2008; Hečimović *et al.*, 2011; Lecoultre *et al.*, 2014). As reported by Farah & Donangelo (2006), roasting conditions would be able to quite completely break down chlorogenic acids to phenolic derivatives, reducing up to 94% the initial CGA content. 3-CQA together with 5-CQA accounted for 55% of CGA in unroasted coffee (Table 8). These caffeoylquinic acids have actually been reported to represent the most abundant CGA in coffee (Farah & Donangelo, 2006; Y. Kim, Keogh, & Clifton, 2016). A 30-fold decrease in 3-CQA concentration was observed in the very dark sample when compared to unroasted coffee, while a 6-fold reduction was found for the 5-CQA, suggesting a different sensitivity to thermal degradation of these compounds, despite they present analogous chemical structures (Dawidowicz & Typek, 2017). The prominent decrease in 3-CQA, which was associated with a less pronounced decrease in 5-CQA content, could also be attributed to a partial isomerization from 3- to 5-position. Changes of CGA structure typically occur when coffee is exposed to high temperature, i.e. during roasting (Farah & Donangelo, 2006).

To obtain a standard indicator for rating the efficacy of coffee extracts in inhibiting α-glucosidase, the IC_{50} (half-maximal inhibitory concentration) of medium, dark and very dark roasted coffee extracts were computed (Table 8).

Table 8. IC_{50} (half-maximal inhibitory concentration) and acarbose equivalents of coffee extracts, 3-caffeoylquinic acid (3-CQA) and 5-caffeoylquinic acid (5-CQA).

Inhibitor	IC_{50} (mg/mL)	Acarbose equivalents ($IC_{50\ Acarbose}/IC_{50\ inhibitor}$)
Medium roasted coffee extract	2.92	0.03
Dark roasted coffee extract	3.24	0.03
Very dark roasted coffee extract	2.41	0.04
3-CQA	0.86	0.11
5-CQA	0.44	0.21
Acarbose	0.09	1

Table 8 also shows the IC_{50} of 3-CQA, 5-CQA and acarbose solutions as well as the acarbose equivalents of the enzyme inhibitors. Roasted coffee extracts resulted 2.8- to 3.8-fold, 5.5- to 7.4-fold and 25- to 33-fold less effective in inhibiting α-glucosidase than 3-CQA, 5-CQA and acarbose, respectively. Thus, in the case of the very dark roasted sample, 7500 mg/d

would produce an α-glucosidase inhibitory effect analogous to that carried out by 300 mg/d acarbose, which is the maximum acarbose dose recommended to adults (Chiasson *et al.*, 2013). Considering that the very dark coffee extract had a 3.3 ± 0.2% dry matter, a cup of espresso coffee (25 mL) would provide 750 mg dry matter. This means that 10 cups of espresso coffee would be required to produce an acarbose-like effect. Nonetheless, studies considering the effect of digestion and absorption of bioactive compounds, as well as validation *in vivo* trials are required to support these speculations.

The complex composition expected in coffee extracts could explain their higher IC_{50}, when compared to that calculated for 3-CQA and 5-CQA. It is noteworthy that no significant relationship ($p > 0.05$) between the phenolic content of coffee extracts (Table 8) and the enzyme inhibitory activity (Figure 8) was found. In particular, even if the highest 3-CQA and 5-CQA concentrations were found in green coffee, the latter did not exert any inhibitory effect against α-glucosidase. Actually, green coffee composition is dominated by carbohydrates (almost 60% dry weight), followed by lipids (8-18% dry weight) and proteins (9-16% dry weight), while phenolic compounds account for 6 to 10% of the dry matter (Ludwig, Clifford, *et al.*, 2014). As already stated, phenolic compounds are well known to undergo to remarkable changes during roasting and they can be incorporated into melanoidin structures (Borrelli *et al.*, 2002; Moreira *et al.*, 2012). Despite the high phenolic content was considered one of the main factors affecting the ability of coffee to reduce diabetes risk (Nieber, 2017), phenols might thus not be the only candidates influencing the α-glucosidase inhibitory capacity of roasted coffee extracts; other compounds, namely melanoidins that are formed during roasting, may also play such a role (Ludwig, Clifford, *et al.*, 2014). A positive linear correlation was actually found between the inhibitory activity and browning development ($p < 0.005$; $R^2 = 0.899$) as well as weight loss ($p < 0.05$; $R^2 = 0.958$).

3.1.3.2 Effect of roasting on the *in vitro* inhibitory activity of coffee fractions against α-glucosidase

To reduce matrix complexity, medium, dark and very dark roasted coffees were fractionated by gel permeation chromatography and fractions containing LMW, IMW and HMW compounds were analyzed for their α-glucosidase inhibitory activity. Green extract was not fractionated, since it did not show any inhibitory activity in the range of interest (Figure 8). Table 9 shows the percentage of each fraction obtained from the medium, dark and very dark roasted coffee extracts, which was calculated by integrating peak areas in the gel permeation chromatograms.

Table 9. High, intermediate and low molecular weight fractions (HMW, IMW, LMW) content (%) in medium, dark and very dark roasted coffee extracts.

Fraction	Molecular weight (Da)	Composition*	Content (%)		
			Medium	Dark	Very dark
HMW	>100	Melanoidins	3	12	8
IMW	15-60	Intermediate-molecular-weight compounds	61	38	2
LMW	1-6	Phenolic compounds	36	50	90

*according to Borrelli *et al.* (2002).

Only a negligible amount (3%) of HMW compounds (i.e. melanoidins) was found in the medium roasted coffee extract, being these more abundant in the dark roasted sample (12%). Further roasting (i.e. very dark roasting degree) produced a 4% reduction in HMW fraction. Although melanoidins are well known to develop during roasting, prolonged and intensive treatments can induce their fragmentation (Borrelli *et al.*, 2002). This evolution is supported by the observed increase of LMW fraction from 36% in the medium roasted coffee extract to 90% in the very dark roasted one, and the concomitant reduction in the IMW compounds from 61% in the medium roasted coffee extract to 2% in the very dark roasted sample. This reduction can be attributed to the degradation of highly thermosensitive phenolic compounds (Hečimović *et al.*, 2011).

Medium roasted coffee fractions did not show any activity in the whole range of analyzed concentrations, up to 10 mg/mL (data not shown), despite a slight inhibition was observed for the correspondent extract (Figure 8). This suggests a synergistic effect among the compounds contained in the medium roasted coffee, leading to a higher inhibitory activity observed for the extract than for the individual fraction. Figure 9 shows the α-glucosidase inhibitory activity of dark and very dark roasted coffee fractions as a function of their concentration.

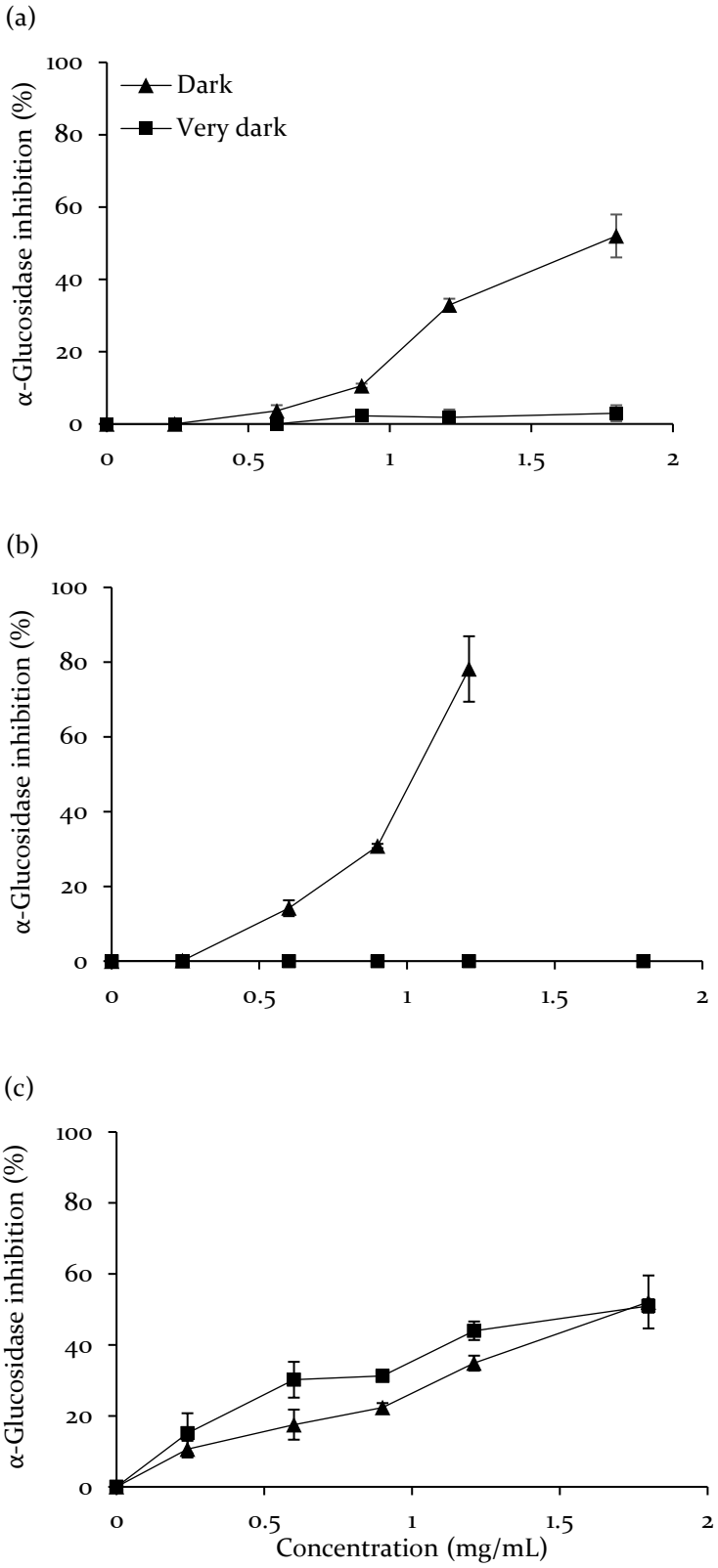


Figure 9. *In vitro* inhibitory activity against α-glucosidase of high (a), intermediate (b) and low (c) molecular weight fractions (HMW, IMW, LMW) of dark and very dark coffee extracts, as a function of their concentration.

HMW, IMW and LMW fractions inhibited α-glucosidase to a different extent. A difference in the inhibitory capacity was also detected within a same fraction, i.e. a same class of compounds (Borrelli *et al.*, 2002), when submitted to different roasting intensities (i.e. dark and very dark). In particular, prolonging roasting from dark to very dark degree suppressed the inhibitory activity of HMW fraction (Figure 9a). Analogously, the inhibitory activity of IMW fraction (Figure 9b) was absent in the very dark roasted sample, whereas it increased exponentially with concentration in the dark processed coffee sample (e.g. 80% inhibition with 1.25 mg/mL). Finally, LMW fraction accounted for lower differences in α-glucosidase inhibitory activity upon roasting between dark and very dark samples than HMW and IMW fractions (Figure 9c). These results suggest that roasting leads not only to changes in the fraction content of coffee extracts (Table 9), but also to qualitative modifications within the fractions. To get an insight into the CGA composition of coffee fractions, Figure 10 shows the concentrations of 3-CQA, 5-CQA and other CGA (calculated as the difference between CGA, and 3-CQA and 5-CQA) in the HMW, IMW and LMW fractions of medium, dark and very dark roasted coffee.

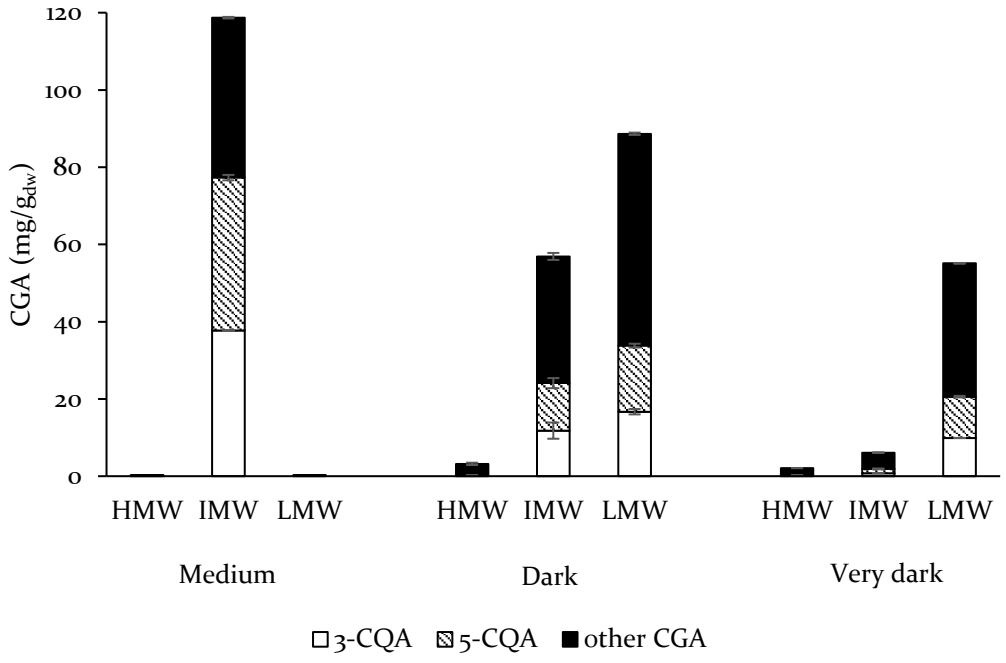


Figure 10. 3-Caffeoylquinic acid (3-CQA), 5-caffeoylquinic acid (5-CQA) and other chlorogenic acid (other CGA) contents of high, intermediate and low molecular weight fractions (HMW, IMW, LMW) of medium, dark and very dark coffee extracts.

CGA, 3-CQA and 5-CQA could not be quantified in the HMW fraction of the medium roasted coffee, while only negligible amounts were found in the dark and very dark HMW fractions. This was expected, since the HMW fraction is mainly represented by melanoidins

(Borrelli *et al.*, 2002). As observed in the case of coffee extracts (Table 8), the CGA content in the IMW fraction underwent a progressive decrease upon roasting (Figure 10), that resulted particularly pronounced in the case of 3-CQA (47-fold decrease). Surprisingly, CGA were not quantifiable in the LMW fraction of the medium roasted coffee extract. To this regard, it should be noticed that, despite LMW is expected to contain mainly phenolic compounds, it could also include other low molecular weight compounds (Perrone, Donangelo, & Farah, 2008). The low concentration of CGA in the LMW fraction of the medium roasted coffee extract could also depend on the incorporation of CGA in melanoidins at the beginning of the roasting process (e.g. medium roasting degree), as reported by Perrone, Farah, and Donangelo (2012). These authors also showed that the melanoidin-CGA complexes are degraded upon further roasting. This could explain the observed increase in 3-CQA, 5-CQA and other CGA concentrations in the LMW fraction upon dark roasting (Figure 10). Further roasting (i.e. very dark) produced up to 40% decrease in CGA concentration, due to the thermal degradation of these compounds, as observed in the case of coffee extracts (Table 8) and reported in the literature (Fujioka & Shibamoto, 2008; Hečimović *et al.*, 2011).

As in the case of coffee extracts (Table 8 and Figure 8), no relation (p>0.05) was found between the phenolic composition of coffee fractions (Figure 10) and their α-glucosidase inhibitory effect (Figure 9).

The latter is probably exerted not only by phenolic compounds or melanoidins, as reported in the literature (Iwai *et al.*, 2012; Johnston *et al.*, 2003; Ludwig, Clifford, *et al.*, 2014; Van Dijk *et al.*, 2009), but might rely on their interaction as well as on other bioactive compounds, whose structural changes, induced by technological interventions, are well known to affect their functional properties (Esquivel & Jiménez, 2012; Vitaglione, Fogliano, & Pellegrini, 2012).

3.1.3.3 Inhibitory mechanisms of roasted coffee extracts and their fractions against α-glucosidase

α-Glucosidase inhibition mechanisms of coffee and coffee components have been little studied (S. D. Kim, 2015). In the present research, assays were performed to investigate the inhibition mechanism against α-glucosidase of coffee extracts and fractions. Enzymatic activity data were elaborated to obtain the Michaelis-Menten plot that describes the relation between the reaction rate (v) and the substrate concentration S. As an example, Figure 11a shows the relationship between the reaction rate and the substrate concentration in the

presence of increasing concentrations of the very dark roasted coffee extract, taken as an inhibitor *I*.

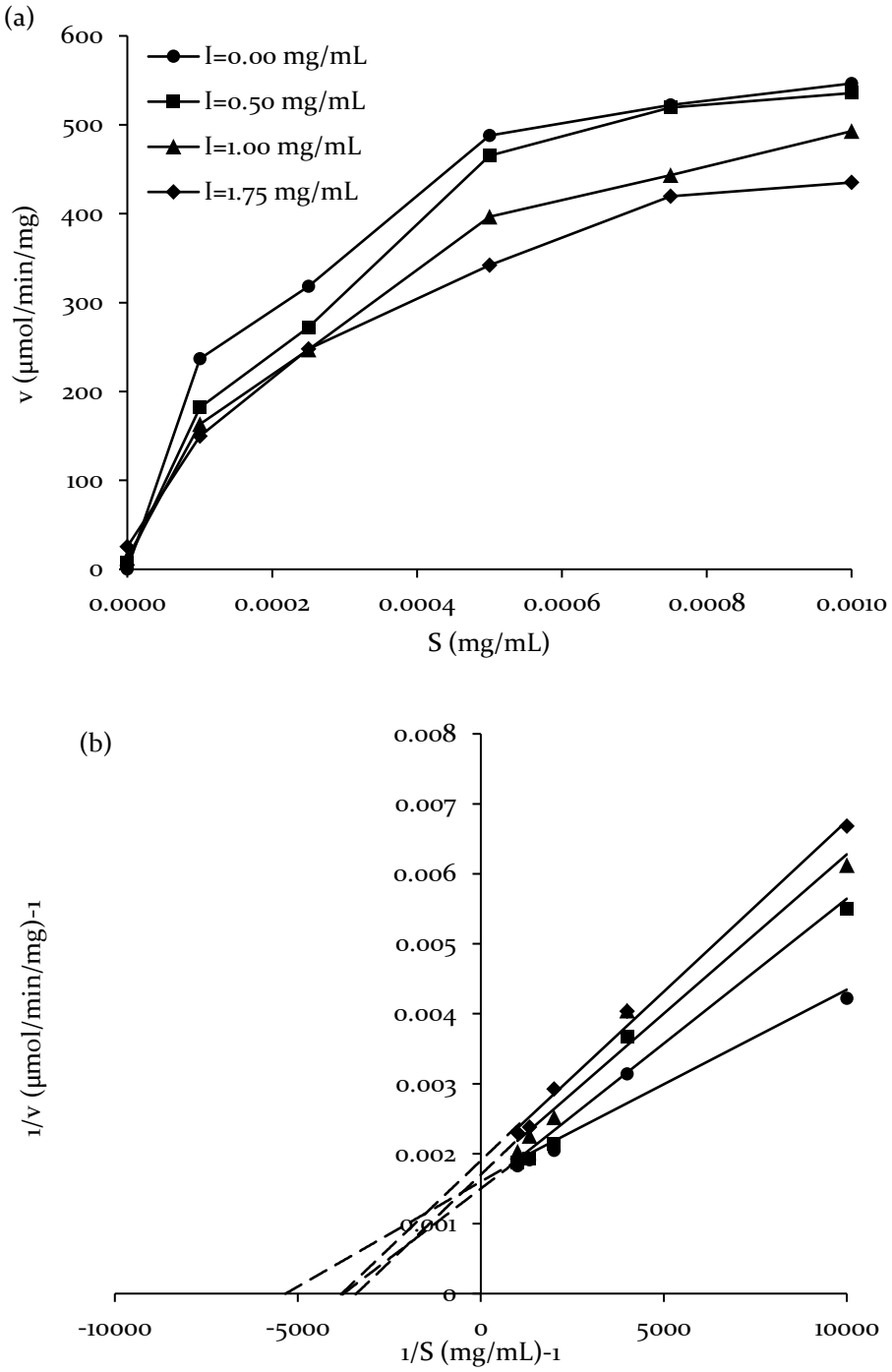


Figure 11. Michaelis-Menten (a) and Lineweaver-Burk (b) plots, in the absence and in the presence of increasing concentration of very dark roasted coffee extract (inhibitor, I). Data fitting: continuous line, estimates: dotted line.

As expected, *v* increased as the substrate concentration increased, approaching a plateau at high concentrations. In our experimental conditions, the evolution of the reaction rate of enzymatic activity as a function of the substrate concentration was comparable in the

absence and in the presence of the coffee extract. However, the reaction rate decreased as the inhibitor concentration increased, indicating an inhibitory activity of the coffee sample. Similar effects were found also for the medium and dark roasted coffee extracts as well as for the fractions obtained from coffees with dark and very dark degree of roast (data not shown). To properly describe the inhibition mechanism, V_{max} and k_m or V_{max^*} and k_{m^*} values, in the absence or in the presence of inhibitor respectively, were computed from the double-reciprocal Lineweaver-Burk plot that describes the relationship between the inverse of the reaction rate (1/v) and the inverse of the substrate concentration (1/S). Figure 11b shows the Lineweaver-Burk plot relevant to the very dark roasted coffee extract. Data regression ($p < 0.05$, $R^2 \geq 0.98$) revealed converging lines intercepting on the y-axis and x-axis (Figure 11b) and provided the reciprocal Vmax and reciprocal km, respectively. The same procedure was applied to all coffee extracts and fractions that presented inhibitory activity, and results were used to compute the kinetic parameters V_{max}, k_m, V_{max^*}, k_{m^*} (Table 10).

Table 10. Kinetic parameters of *in vitro* α-glucosidase activity in the absence and in the presence of increasing concentrations of extract or high, intermediate and low molecular weight fractions (HMW, IMW, LMW) of dark or very dark roasted coffee.

Roasting degree	Sample	I (mg/mL)	V_{max} (µmol/min/mg)	V_{max*} (µmol/min/mg)	k_m (µg/mL)	k_{m*} (µg/mL)	k_i (µg/mL)	k_i' (µg/mL)	Type of inhibition
Dark	Extract	0.00	714	-	0.17	-	1,472	12,167	Mixed-type
		0.50	-	667	-	0.21			
		1.00	-	667	-	0.31			
		1.75	-	625	-	0.36			
	HMW	0.00	833	-	0.50	-	1,260	5,460	Mixed-type
		0.24	-	833	-	0.50			
		0.42	-	769	-	0.62			
	IMW	0.00	625	-	0.13	-	206	-	Competitive
		0.12	-	667	-	0.20			
		0.24	-	667	-	0.27			
	LMW	0.00	625	-	0.13	-	360	-	Competitive
		0.12	-	625	-	0.19			
		0.24	-	625	-	0.19			
Very dark	Extract	0.00	625	-	0.19	-	2,063	14,042	Mixed-type
		1.00	-	588	-	0.29			
		1.75	-	526	-	0.26			
	LMW	0.00	1000	-	0.40	-	420	1,120	Mixed-type
		0.42	-	625	-	0.50			

Both coffee extracts (i.e. dark and very dark) presented an increase from k_m to k_{m*}, and a concomitant decrease from V_{max} to V_{max*} (Table 10). The changes in both kinetic parameters can be attributed to a mixed-type inhibition with k_i and k_i' constants (E. M. Ahmed *et al.*, 2014). The latter typically occurs when the inhibitor binds to a site other than the active one, inducing enzymatic conformational changes and thus reducing substrate affinity for the active site (Robinson, 2015; Shou *et al.*, 2000). A similar behaviour was observed in the case of the very dark LMW and the dark HMW fractions. These results agree with literature findings, since Ghadyale, Takalikar, Haldavnekar, and Arvindekar (2012), and X. J. Hu *et al.* (2013) found a non-competitive mechanism against α-glucosidase for two different plant extracts, i.e. *Cymbopogon martinii* and *Momordica charantia*, respectively. It should be noted that in mixed-type and non-competitive mechanisms the inhibitor binds to both the free enzyme and enzyme-substrate complex. However, non-competitive inhibitor presents the same affinity towards enzyme and enzyme-substrate complex, while mixed-type inhibitor has different affinities. Table 10 also shows the kinetic parameters of the IMW and LMW fractions of the dark coffee. The latter presented k_{m*} higher than k_m, while V_{max} was not affected, suggesting a competitive inhibition. This mechanism was described in the literature by several authors regarding anthocyanins and other polyphenols (McDougall & Stewart, 2005; Sancho & Pastore, 2012). Interestingly, the competitive mechanism also underlies the activity of synthetic α-glucosidase inhibitors, such as acarbose (Matsui *et al.*, 2001; Toeller, 1994).

According to the acquired results, roasting affected not only the inhibitory efficacy of coffee fractions (Figure 9), but also their mechanism (Table 10). Both are expected to depend on the complex composition of coffee matrix, which is known to change upon roasting (Table 8, Table 9, Figure 10).

3.1.4 Conclusions

Roasting affected the *in vitro* α-glucosidase inhibitory activity of coffee. The darker the coffee degree of roast the greater such a property. Gel permeation chromatography of coffee extracts allowed obtaining low to high molecular weight coffee fractions. Although they were nominally representative of phenolic compounds (LMW fraction), phenolic compounds and Maillard reaction intermediates (IMW fraction), and melanoidins (HMW fraction), results showed that increasing roasting time caused changes in their inhibitory activity against the digestive enzyme. This suggests compositional changes occurring within each fraction due to the roasting process. Coffee extracts and their fractions revealed mixed-

type to competitive inhibition mechanisms against α-glucosidase, which are consistent with the complexity of samples composition, as well as with literature findings relevant to several vegetable matrices.

Although the results of this study are far from clearing-up the effect of coffee consumption on diabetes, nevertheless they indicate that coffee negatively affects the *in vitro* activity of one key enzyme in the carbohydrate digestive process, suggesting the potential of coffee and coffee derivatives as adjuvants for the treatment of type 2 diabetes.

Indeed, further research is required to investigate how coffee antidiabetic potential is affected not only by processing but also by formulation, considering eventual interactions occurring among food components. In addition, transformations occurring during the digestion process should be considered as potentially affecting coffee functionality. This approach could represent a starting point for the development of integrated strategies to treat and prevent type 2 diabetes. *In vivo* validation of this activity would open the possibility to make claims about coffee preventing type 2 diabetes.

3.2 α-Glucosidase inhibitory capacity of *in vitro* digested coffee as affected by roasting

3.2.1 Aim of the study

The aim of this study was to further investigate the effect of roasting on the ability of coffee to inhibit α-glucosidase after *in vitro* digestion and to get a deeper insight into the compounds potentially involved in such an activity.

3.2.2 Material and methods

3.2.2.1 Sample preparation

Coffee samples were prepared as reported in paragraph 3.1.2.1. An additional sample was considered, i.e. light roasted coffee, which was obtained after 10 min roasting at 200 °C.

3.2.2.2 Weight loss and total solid content determinations

Sample weight loss upon roasting and total solid content were determined as described in paragraph 3.1.2.3.

3.2.2.3 Color analysis

Color analysis was carried out as described in paragraph 3.1.2.4.

3.2.2.4 *In vitro* digestion

In vitro digestion was carried out according to the protocol proposed by Minekus *et al.* (2014) and as described in detail in paragraph 2.1.2.9, except for some changes. In particular, pancreatin was used instead of lipase during the intestinal phase. In addition, at the end of the intestinal phase, samples were ultrafiltered (10 kDa, Vivaspin 500, Sartorius, Varedo, Italy) by centrifugation at 13,000 ×*g* for 25 min at 4 °C (Hittich MIKRO 20 Centrifuge, Tuttlingen, Germany). The ultrafiltered sample was considered as the bioaccessible fraction (Tagliazucchi *et al.*, 2012). Bioaccessibility was determined as the ratio between the concentration of the compound in the digested sample and that in the undigested sample and results were expressed as a percentage.

3.2.2.5 Chlorogenic acid quantification and bioaccessibility computation

Chromatographic quantification of chlorogenic acids (CGA) was performed on undigested and digested samples following the method proposed by Mills, Oruna-Concha, Mottram, Gibson, and Spencer (2013). An HPLC pump (LC-10AT VP, Shimadzu Corporation, Kyoto, Japan) equipped with a diode array detector (SPD-10 AT VP, Shimadzu Corporation, Kyoto, Japan) and with an inverse phase apolar C18 column (5 µm, 250 x 4.6 mm, Alltima, Lokeren, Belgium) was used. The injection valve (Rheodyne, Sigma-Aldrich, Milano, Italy) was equipped with a 20 µL plastic loop and samples were injected using a syringe (SGE LC, 100 µL, FN). The elution was carried at a flow rate of 1 mL/min in gradient mode using 5% methanol (Sigma-Aldrich, Milano, Italy) and 95% water containing 0.1% HCl 5 N (solvent A), and 50% acetonitrile (Sigma-Aldrich, Milano, Italy) and 50% water containing 0.1% HCl 5 N (50%) (solvent B) as mobile phase. Gradient was set as follows: solvent A was held at 95% for the first 5 min, decreased to 50% and held at this level up to 40 min; then decreased further to 0% and held up to 59.9 min; finally, 95% solvent A was reached and held up to 60 min. The detection was conducted at 320 nm. Quantification was carried out using 3-CQA and 5-CQA as external standards. Calibration curves were linear (R^2 > 0.995) in the 2.0 to 200.0 mg/L concentration interval. Peak integration was performed by using Polyview 2000 software (Ver. 5.3, Varian, Texas, USA).

3.2.2.6 α-Glucosidase inhibition assay

The inhibitory activity of digested coffee brew was assessed as previously described (paragraph 3.1.2.6).

3.2.2.7 Browning measurement

The absorbance at 280, 360 and 420 nm was measured (UV-2501PC, UV-VIS Recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) in suitably diluted undigested and digested coffee and represented a non-specific marker of Maillard reaction (Delgado-Andrade, Morales, Seiquer, & Pilar Navarro, 2010). In particular, early non-colored compounds were monitored at 280 nm, the pool of more advanced ones at 360 nm, and final high molecular weight compounds, namely melanoidins, at 420 nm. Absorbance values were then adjusted based on the dilution factor, i.e. 4 and 2 for undigested and digested samples, respectively, to obtain Maillard reaction indices.

3.2.2.8 Statistical analysis

Data were reported as mean ± standard deviation of at least three measurements on two replicated samples. Analysis of variance (ANOVA) was performed with significance level set to $p < 0.05$; the Bartlett procedure was used to test homogeneity of variances, using R software, version 3.4.3 (The R Foundation for Statistical Computing, 2018). Linear regression analysis was performed by using Microsoft Excel 2016. The goodness of fitting was evaluated based on visual inspection of residual plots and by the calculation of R^2 and p.

3.2.3 Results and discussion

Roasted coffee was classified as light, medium, dark and very dark based on weight loss (Clarke, 1987) and was characterized for total solid content and color (Table 11). As expected, these parameters changed upon roasting following a similar trend as reported in the literature (Moreira *et al.*, 2012; Opitz *et al.*, 2014; Vargas-Elías *et al.*, 2016) and as previously observed (Table 8).

Table 11. Weight loss, total solid content and color of ground Robusta coffee beans with different degree of roast. Means in the same column indicated by a common letter (a-d) are not significantly different ($p > 0.05$).

Degree of roast	Weight loss (%)	Total solid content (%)	L*	Hue angle ($tan^{-1}b^*/a^*$)
Unroasted	0	90.6 ± 0.4 ^{d}	58.1 ± 0.4 ^{b}	81.0 ± 0.4 ^{a}
Light	5	93.8 ± 0.0 ^{c}	61.4 ± 0.8 ^{a}	80.8 ± 0.7 ^{a}
Medium	7	95.6 ± 0.5 ^{b}	62.7 ± 1.7 ^{a}	77.5 ± 0.5 ^{b}
Dark	10	97.5 ± 0.0 ^{a}	49.9 ± 1.4 ^{c}	67.7 ± 1.1 ^{c}
Very dark	12	98.7 ± 0.1 ^{a}	42.3 ± 0.6 ^{d}	61.0 ± 0.7 ^{d}

Differently roasted coffee beans were then used to prepare coffee extracts, which were *in vitro* digested and analyzed for their ability to inhibit α-glucosidase (Figure 12). A control represented by the digestive mixture not containing coffee brew was also tested and did not present any inhibitory effect in the whole range of analyzed concentrations (data not shown). The efficacy of samples in inhibiting the enzyme was rated by estimating the half-maximal inhibitory concentration (IC_{50}) and the acarbose equivalent (AE), based on acarbose IC_{50} (Table 8), and results are shown in Table 12.

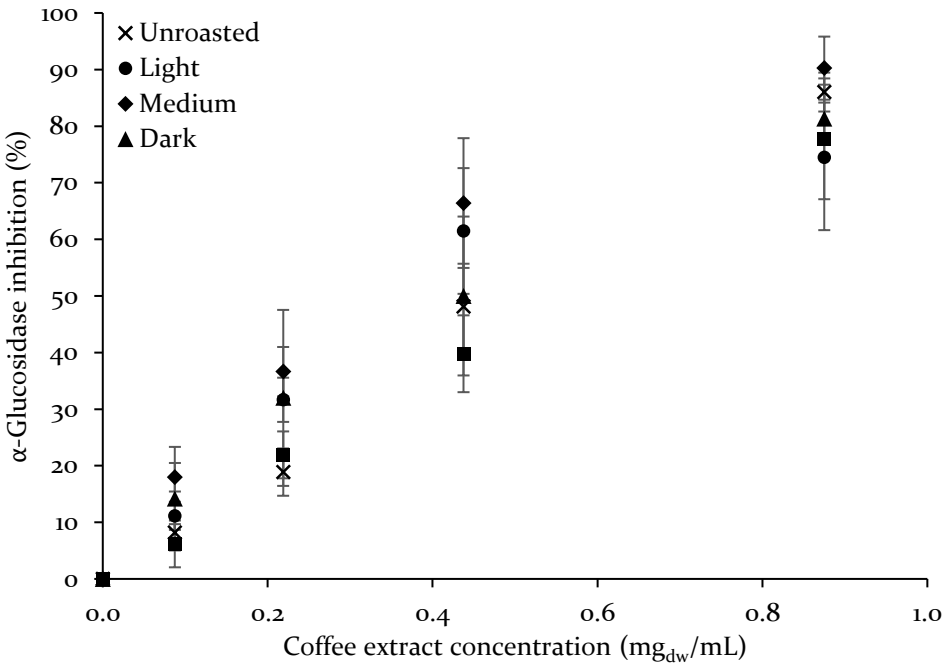


Figure 12. Inhibitory activity against *α*-glucosidase of unroasted, light, medium, dark and very dark roasted coffee extracts submitted to *in vitro* digestion, as a function of their concentration.

Table 12. Half-maximal inhibitory concentration (IC_{50}) and acarbose equivalent (AE) of digested coffee extracts.

Inhibitor	IC_{50} (mg/mL)	AE
Unroasted	0.50	0.18
Light	0.49	0.18
Medium	0.40	0.22
Dark	0.49	0.19
Very dark	0.56	0.16

All digested coffee extracts inhibited *α*-glucosidase in a concentration dependent manner (Figure 12). Surprisingly no differences were observed among samples obtained from coffee with a different degree of roast. To this regard, it should be noticed that in the previous study an increase in the ability of coffee to inhibit *α*-glucosidase as the roasting degree increased was evidenced (Figure 8). Since this study was carried out on the undigested coffee extract, it can be gathered that digestion can induce changes in the matrix composition, determining a different performance in terms of functional properties.

Although no differences depending on the degree of roast were found in the ability to inhibit *α*-glucosidase, interestingly digested coffee extracts (Table 12) were much more effective than the undigested samples in inhibiting the enzyme. In fact, an IC_{50} ranging from 2 to 3

mg/mL was observed for undigested roasted coffees (Table 8), whereas digested samples presented on average a 6-fold lower value, resulting thus six times more effective. Considering AE values, it is noteworthy that digested coffees presented an inhibitory efficacy corresponding on average to the 20% of that reported for acarbose (Table 12), that is one of the drugs commonly used to treat type 2 diabetes. In addition, the inhibitory efficacy of digested coffees was comparable to that previously found for 5-CQA (0.44 mg/mL, Table 8), that is one of the most abundant phenolic compounds found in coffee (Yilmaz & Kolak, 2017) and is well known for its antidiabetic properties (Xu *et al.*, 2015).

Based on the dry weight of coffee brew (35 mg/mL), the average volume of an espresso cup (25 mL) and the daily recommended dose of acarbose (300 mg), it is possible to roughly estimate the number of cups that would be required to obtain an "acarbose-like" effect. Considering the AE (Table 12), 1,666 mg dry weight of coffee would be required to match the acarbose dose. One cup of coffee would provide 875 mg of coffee dry matter. Thus, nearly 2 cups of coffee, that is a reasonable daily consumed amount, would produce an acarbose-like effect. Similar considerations were previously reported but considered the undigested coffee and nearly 10 cups were computed as necessary to produce an acarbose-like effect. It is thus clear that digestion increased the antidiabetic potential of coffee, although *in vivo* trials are still required to support these speculations. In addition, the interaction effects deriving from the co-ingestion with other food matrices, such as sugar and milk, still need to be considered.

To get an insight into changes occurring in phenolic composition not only upon roasting but also after *in vitro* digestion, undigested and digested coffee samples were analyzed for CGA content (Figure 13).

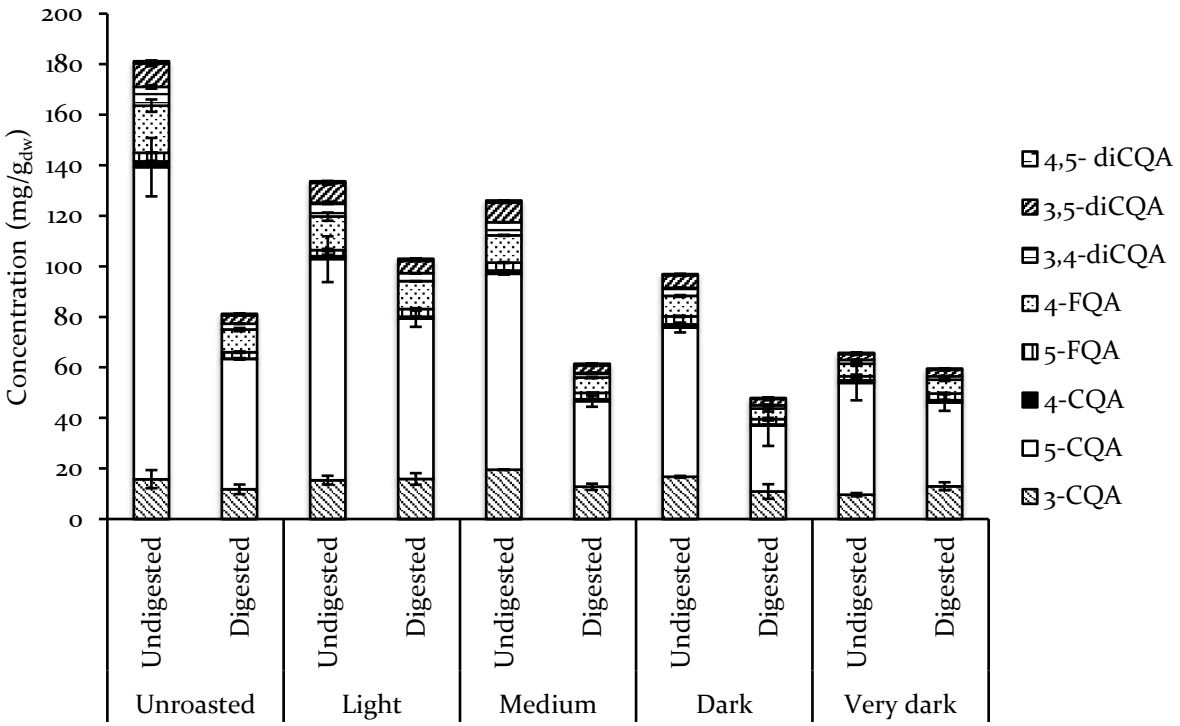


Figure 13. Concentration of 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA), 5-caffeoylquinic (5-CQA), 5-feruloylquinic (5-FQA) and 4-feruloylquinic (4-FQA), 3,4-di-caffeoylquinic (3,4-di-CQA), 4,5-di-caffeoylquinic (4,5-di-CQA) and 3,5-di-caffeoylquinic (3,5-di-CQA) acids in coffee before (Undigested) and after (Digested) *in vitro* digestion.

CGA can be clustered into three main groups. In particular, di-caffeoylquinic acids (di-CQAs), feruloylquinic acids (FQAs) and caffeoylquinic acids (CQAs), with 5-O-caffeoylquinic acid (5-CQA) being the most abundant, in agreement with the literature (Michael N. Clifford & Knight, 2004). It can be noticed that 5-CQA was actually the most abundant chlorogenic acid in all samples, both before and after *in vitro* digestion, always accounting for more than 50% of overall CGA.

As reported in Figure 13, the CGA concentration in undigested samples almost progressively decreased as the roasting degree increased, in agreement with previous findings (Table 8). A 3-fold reduction in total CGA was observed when the highest roasting intensity was applied, with 5-CQA being one of the compounds most susceptible to degradation.

As shown in Figure 13, digestion produced a decrease in CGA concentration, accounting for almost 50% in the unroasted sample, in agreement with data reported by other authors for coffee (López-Froilán *et al.*, 2016; Olthof, Hollman, & Katan, 2001; Podio *et al.*, 2015), as well as for other foods such as tea (Record & Lane, 2001) and fruit juices (Cilla *et al.*, 2009). Such a reduction could be due not only to the activity of digestive enzymes but also to the low stability of phenolic compounds to pH modification during the digestion process. In

addition, phenolic compounds can undergo several reactions during *in vitro* digestion, such as polymerization, epimerization, and auto-oxidation, as well as complexation with metal ions present in the digestive mixture (Rodríguez-Roque, Rojas-Graü, Elez-Martínez, & Martín-Belloso, 2014).

In addition, digestion almost neutralized the differences produced by roasting. In fact, all digested samples contained a comparable amount of total CGA, independently from the roasting degree and corresponding to the 30-50% of the initial concentration found in the undigested unroasted coffee.

Nonetheless, the phenolic profile of digested coffee resulted analogous to that of the undigested sample, with 5- and 3-CQA still representing the majority of overall CGA (Figure 13).

Based on the concentration of CGA before and after digestion (Figure 13), their bioaccessibility was computed (Table 13).

Table 13. Bioaccessibility (%) of 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA), 5-caffeoylquinic (5-CQA), 5-feruloylquinic (5-FQA) and 4-feruloylquinic (4-FQA), 3,4-di-caffeoylquinic (3,4-di-CQA), 4,5-di-caffeoylquinic (4,5-di-CQA) and 3,5-di-caffeoylquinic (3,5-di-CQA) acids, and total chlorogenic acids (Total CGA) in coffee beverages with different degree of roasting. Means in the same column indicated by a common letter (a–c) are not significantly different ($p > 0.05$).

Sample	3-CQA	5-CQA	4-CQA	5-FQA	4-FQA	3,4-di-CQA	3,5-di-CQA	4,5-di-CQA	Total CGA
Unroasted	75 ± 12 ^{b}	42 ± 0 ^{c}	3 ± 0 ^{c}	75 ± 8 ^{b}	489 ± 3 ^{a}	31 ±1 ^{b}	36 ±0 ^{b}	75 ±5 ^{bc}	45 ±1 ^{c}
Light	103 ± 15 ^{ab}	73 ± 4 ^{ab}	65 ± 6 ^{ab}	128 ± 11 ^{a}	83 ± 1 ^{ab}	58 ± 2 ^{b}	62 ±1 ^{b}	139 ± 0.2 ^{a}	77 ±4 ^{ab}
Medium	66 ± 6 ^{b}	44 ± 3 ^{bc}	65 ± 13 ^{ab}	76 ± 3 ^{b}	57 ± 3 ^{bc}	35 ± 5 ^{b}	39 ±3 ^{b}	74 ± 10 ^{bc}	49 ±4 ^{bc}
Dark	65 ± 17 ^{b}	44 ± 14 ^{c}	32 ± 7 ^{cb}	68 ± 17 ^{b}	52 ± 14 ^{c}	39 ±11 ^{b}	53 ±11 ^{b}	71 ±4 ^{c}	49 ±12 ^{bc}
Very dark	135 ± 17 ^{a}	75 ± 8 ^{a}	86 ± 19 ^{a}	155 ± 18 ^{a}	112 ± 10 ^{a}	90 ±12 ^{a}	109 ±14 ^{a}	93 ± 9 ^{b}	91 ± 8 ^{a}

No linear relationship was found between CGA bioaccessibility and degree of roasting, although the very dark roasted coffee had the highest bioaccessibility value (Table 13). However, when examining these results together with data relevant to concentration (Figure 13), it is clear that roasting reduced the concentration of CGA available for intestinal absorption. It can be thus hypothesized that even though roasting induced the degradation of a part of CGA, those surviving to roasting were also able to last through digestion, thus contributing to the bioaccessibility.

It should be noticed that although CGA concentration overall decreased upon roasting, the ability of coffee extracts to inhibit α-glucosidase was enhanced after digestion (Table 12). In other words, digestion produced an opposite effect on these features: CGA were overall reduced, while the ability to inhibit α-glucosidase was improved as compared to the undigested samples (Figure 8 and Table 8), bolstering the hypothesis of other compounds involved in the antidiabetic effect of coffee.

Other compounds actually formed upon roasting, due to the occurrence of Maillard reaction. Even though their structure and the mechanisms behind their formation are still uncertain (Moreira *et al.*, 2012), these compositional changes induce color changes and can be thus monitored through spectrophotometric measurements. Table 14 shows the Maillard reaction indices revealing the presence of early non-colored compounds (monitored at 280 nm), more advanced Maillard reaction products (monitored at 360 nm) and advanced high molecular weight compounds (monitored at 420 nm) in undigested and digested coffees (Bekedam, Schols, Van Boekel, & Smit, 2006; Delgado-Andrade *et al.*, 2010).

Table 14. Maillard reaction indices relevant to compounds monitored at 280, 360 and 420 nm. Lowercase letters (a-c) indicate significant differences ($p < 0.05$) among differently roasted samples, uppercase letters (A-B) indicate significant differences ($p < 0.05$) between digested and undigested samples.

Sample	Undigested			Digested		
	280	360	420	280	360	420
Unroasted	5.32 ± 0.06 ^{a,A}	4.72 ± 0.06 ^{ab,A}	1.95 ± 0.09 ^{c,A}	1.86 ± 0.56 ^{a,B}	1.83 ± 0.61 ^{a,B}	1.89 ± 0.62 ^{a,A}
Light	4.21 ± 0.27 ^{b,A}	3.96 ± 0.03 ^{c,A}	2.18 ± 0.08 ^{c,A}	2.05 ± 0.13 ^{a,B}	1.97 ± 0.21 ^{a,B}	1.97 ± 0.28 ^{a,A}
Medium	5.12 ± 0.05 ^{a,A}	4.86 ± 0.03 ^{a,A}	2.16 ± 0.05 ^{c,A}	2.06 ± 0.01 ^{a,B}	2.02 ± 0.01 ^{a,B}	1.97 ± 0.11 ^{a,A}
Dark	4.87 ± 0.02 ^{a,A}	4.26 ± 0.31 ^{bc,A}	4.16 ± 0.28 ^{b,A}	2.63 ± 0.67 ^{a,A}	2.57 ± 0.66 ^{a,A}	2.55 ± 0.64 ^{a,A}
Very dark	5.20 ± 0.06 ^{a,A}	4.86 ± 0.08 ^{a,A}	4.78 ± 0.03 ^{a,A}	2.04 ± 0.23 ^{a,B}	1.99 ± 0.24 ^{a,B}	2.01 ± 0.24 ^{a,B}

The indices at 280 and 360 nm did not differ among undigested samples, indicating the presence of similar amounts of early and intermediate Maillard reaction compounds. On the contrary, the Maillard reaction index at 420 nm progressively increased with the increase in the degree of roast, more than doubling in the very dark roasted sample. Such color change, also highlighted by color analysis of ground coffee (Table 11), can be attributed to changes in the composition occurring during roasting that lead to melanoidin development (Borrelli *et al.*, 2002). These molecules are formed through the condensation of smaller compounds, including CGA (Bekedam, Schols, Van Boekel, & Gerrit, 2008). These results are in agreement with previous findings, showing a decrease in CGA concentration and an increase in the browning index of coffee (Table 14). In the previous study we also observed a positive linear correlation between the inhibitory activity and browning development, suggesting that melanoidins could play a critical role towards α-glucosidase inhibition.

It can be noticed that overall Maillard reaction indices significantly decreased upon digestion (Table 14). In addition, these indices did not differ as a function of the roasting degree in digested samples, analogously to what observed for CGA (Figure 13). This further suggests that the digestion process flattened the differences induced by roasting. Actually, the inhibitory activity against α-glucosidase carried out by digested samples (Figure 12) was comparable independently from the degree of roast. This might suggest that nor CGA, neither melanoidins alone could explain the ability of coffee to inhibit the enzyme. A complex interaction among molecules produced upon digestion, such as quinic acid and chlorogenic acid lactones (Dawidowicz & Typek, 2017), as well as molecules deriving from the fragmentation of Maillard reaction products (Borrelli *et al.*, 2002), could be responsible for the antidiabetic effect of coffee brews.

3.2.4 Conclusions

Results reported in the present study highlighted that although roasting induced compositional changes in coffee, digestion flattened these differences. Similarly, roasting-dependent modifications observed in the antidiabetic potential of coffee, i.e. its ability to inhibit α-glucosidase, disappeared upon digestion. Nonetheless, digested coffee extracts exhibited a higher efficacy towards α-glucosidase inhibition as compared to undigested samples, confirming the potential of coffee use as a tool for dietetic approaches designed to manage type 2 diabetes.

Still, the compounds and the mechanisms behind such an effect remain partially unrevealed and further research is required to delve into the antihyperglycemic potential of coffee.

This research also pointed out that although processing could produce significant changes in the composition of a food matrix, it should be kept in mind that also digestion plays a critical role in modifying the chemical composition of food, especially if considering bioactive compounds. When designing functional foods, it is thus crucial to concomitantly take into account the effects of processing and those of digestion, to successfully deliver the desired outcomes at gastrointestinal level.

3.3 Fat concentration and high-pressure homogenization affect chlorogenic acid bioaccessibility and α-glucosidase inhibitory capacity of milk-based coffee beverages

3.3.1 Aim of the study

The aim of this study was to investigate the effect of coffee brew formulation (with and without milk at different fat concentrations) and HPH on CGA bioaccessibility and inhibitory effect against α-glucosidase activity upon *in vitro* digestion. The results here presented have been published:

- ✓ Alongi, M., Calligaris, S., Anese, M. (2019). Fat concentration and high-pressure homogenization affect chlorogenic acid bioaccessibility and α-glucosidase inhibitory capacity of milk-based coffee beverages. *Journal of Functional Foods, 58*, 130-137.

3.3.2 Material and methods

3.3.2.1 Sample preparation and high-pressure homogenization

Coffee brew was prepared by dissolving instant coffee (Nescafè Gran Aroma, Nestlè, Vevey, Swiss) in deionized water (20 mg/mL). Skimmed milk (0.1% fat, w/w) and cream (35% fat, w/w) (Granarolo S.p.A, Bologna, Italy) were purchased on the local market and properly mixed to obtain milk containing 3.6% and 7.1% (w/w) fat, respectively. Coffee brew (50%, w/w) and milk containing 0.1, 3.6 and 7.1% fat (50%, w/w) were then mixed to obtain 10 mg/mL coffee beverages with low, intermediate and high-fat content, respectively. These beverages were homogenized by using a continuous lab-scale high-pressure homogenizer (Panda Plus 2000, GEA Niro Soavi, Parma, Italy) supplied with two PS type homogenization valves with a flow rate of 10 L/h was used to treat 150 mL of sample. The first valve was the actual homogenization stage and was set at 50, 100 and 150 MPa. The second valve was set at a constant value of 10 MPa. At the exit of the homogenizer, the samples were forced into a heat exchanger set at 4 °C (GEA Niro Soavi, Parma, Italy) to cool samples to T < 20 °C. Coffee brew and beverages were aliquoted to obtain samples from a same batch to be directly analyzed or to be *in vitro* digested before analyses.

3.3.2.2 *In vitro* digestion

In vitro digestion was carried out according to the protocol proposed by Minekus *et al.* (2014), as described in detail in paragraph 2.1.2.9 and by following the changes described in paragraph 3.2.2.4.

3.3.2.3 Particle physicochemical characterization

The particle size distribution of digested samples was measured as described in paragraph 2.1.2.10, except for sample dilution, which amounted to 1:1000 (v/v).

3.3.2.4 Chlorogenic acid quantification and bioaccessibility

Chromatographic quantification of chlorogenic acids and bioaccessibility computation were carried out as described in paragraph 3.2.2.5.

3.3.2.5 α-Glucosidase inhibition

The inhibitory activity of digested coffee brew and beverages against α-glucosidase was assessed as previously described in paragraph 3.1.2.6. A logarithmic model was used to fit data and to compute the half-maximal inhibitory concentration (IC_{50}).

3.3.2.6 Polynomial equations and statistical analysis

Modeling was aimed at identifying the combination of fat concentration and pressure able to optimize the inhibitory activity against α-glucosidase, by minimizing the IC_{50}. In particular, a 2-factors face-centered central composite design (CCF) was used. The factors considered were fat concentration and pressure and were set at 0.1, 3.6 and 7.1% (w/w) and at 50, 100 and 150 MPa, respectively. The CCF was completed by a central point (combination of the intermediate values of the two factors). All the factorial points were replicated once, while the central point was replicated 3 times (Table 15).

Table 15. Combination of fat concentration and pressure of different runs and experimental results (half maximal inhibitory concentration, IC_{50}) of a two-factors face-centered central composite design.

Run	Fat concentration (%)	Pressure (MPa)	IC_{50} (mg/mL)
1	0.1	50	2.76 ± 0.56
2	0.1	150	0.70 ± 0.03
3	7.1	50	0.92 ± 0.06
4	7.1	150	1.23 ± 0.13
5	0.1	100	0.92 ± 0.11
6	7.1	100	0.50 ± 0.02
7	3.6	50	2.05 ± 0.55
8	3.6	150	1.22 ± 0.14
9	3.6	100	1.18 ± 0.18
10	3.6	100	1.12 ± 0.13
11	3.6	100	0.81 ± 0.05

A second order response surface was fitted to the observed data according to Equation 7.

$$y = B_0 + \sum_{i=1}^{k} B_i x_i + \sum_{i=1}^{k} B_{ii} x_i^2 + \sum_{j>i\geq 1}^{k} B_{ij} x_i x_j \qquad \text{Equation 7}$$

where B_o is a constant, B_i, B_{ii}, and B_{ij} are regression coefficients of the model, x_i and x_j are the independent variables in coded values, and k is the number of factors.

Shapiro-Wilk test was used to evaluate normality of the data, while the possible presence of outliers and the homogeneity of variance were evaluated by residual analysis. The goodness of fit was measured with the adjusted determination coefficient (R^2_{adj}). p-Values for the coefficients of the response surface were defined using standard t-test. Contour plot was drawn to illustrate the effect of the considered factors on the IC_{50}.

Results are averages of three measurements and are reported as mean value ± standard deviation. Analysis of variance (ANOVA) was performed by using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance and Tukey test was used to test for differences between means ($p < 0.05$).

3.3.3 Results and discussion

3.3.3.1 Effect of *in vitro* digestion on coffee brew

The digested coffee brew was tested for its ability to inhibit α-glucosidase (Figure 14).

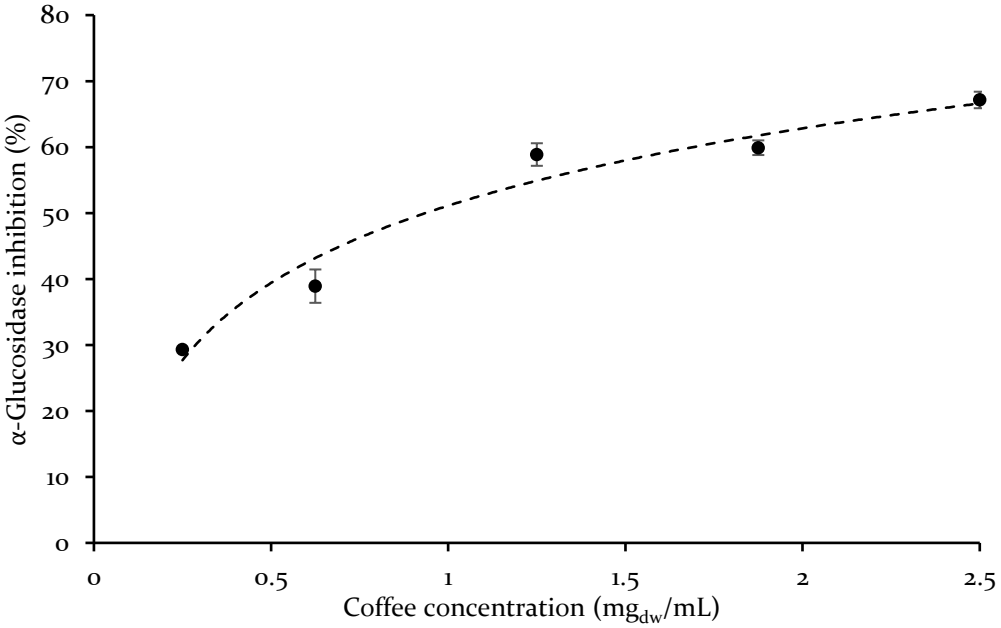


Figure 14. α-Glucosidase inhibitory activity of digested coffee brew as a function of coffee concentration. Data fitting: line, estimates; symbols, experimental data.

As expected based on previously acquired results (Figure 12), the inhibition carried out by digested coffee brew against α-glucosidase increased in a concentration-dependent manner (Figure 14) with an IC_{50} of 0.94 mg_{dw}/mL. The latter was up to 3-fold lower than that reported for undigested coffee beverages (Table 8), indicating again that digested coffee was more effective than the undigested one in inhibiting α-glucosidase.

Coffee brew was also analyzed for chlorogenic acid (CGA) content before and after *in vitro* digestion. Also in this case, two major families of CGA, namely caffeoylquinic acids, i.e. 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA) and 5-caffeoylquinic (5-CQA), and feruloylquinic acids, i.e. 5-feruloylquinic (5-FQA) and 4-feruloylquinic (4-FQA), were identified and quantified (Table 16).

Table 16. Concentration before (Undigested) and after (Digested) *in vitro* digestion of coffee brew, and bioaccessibility of 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA), 5-caffeoylquinic (5-CQA), 5-feruloylquinic (5-FQA), 4-feruloylquinic (4-FQA) and total chlorogenic (Total CGA) acids. Lowercase letters (a-b) indicate significant differences of concentration ($p > 0.05$) between undigested and digested samples, uppercase letters (A) indicate significant differences of bioaccessibility among CGA.

CGA	Undigested (mg/g_{dw})	Digested (mg/g_{dw})	Bioaccessibility (%)
3 CQA	4.24 ± 0.12^{a}	1.22 ± 0.33^{b}	28.85 ± 7.78^{A}
4 CQA	1.57 ± 0.06^{a}	0.39 ± 0.08^{b}	25.09 ± 5.27^{A}
5 CQA	9.99 ± 0.10^{a}	2.14 ± 0.19^{b}	21.40 ± 1.86^{A}
5 FQA	1.41 ± 0.04^{a}	0.39 ± 0.08^{b}	27.72 ± 6.01^{A}
4 FQA	2.74 ± 0.02^{a}	0.54 ± 0.16^{b}	19.53 ± 5.95^{A}
Total CGA	19.95 ± 0.30^{a}	4.68 ± 0.77^{b}	23.46 ± 3.87^{A}

As expected, 5-CQA was the most abundant compound, and, together with 3-CQA, accounted for more than 70% of the overall CGA, in agreement with previous data showing that these compounds represent more than half of coffee CGA (Table 8 and Figure 13). It should be kept in mind that the different magnitude range observed for CGA analyzed in the different studies depends on the different coffee (e.g. Robusta or Arabica, instant or powder) used.

In agreement with previous data (Figure 13) a significant reduction in CGA concentration, up to 80%, was observed upon *in vitro* digestion (Table 16). Still, the phenolic profile of digested coffee resulted analogous to that of the undigested sample, with 5- and 3-CQA representing the 70% of overall CGA (Table 8), as formerly observed (Figure 13). To better understand which was the ratio of phenolic compounds surviving the digestion process and thus available for uptake by the intestinal mucosa (Ferruzzi, 2010), their bioaccessibility was computed (Table 16). The latter accounted for less than 25% and no significant differences were observed among single CGA, that presented a bioaccessibility always lower than 30%, suggesting that all CGA seemed equally susceptible to degradation upon *in vitro* digestion.

It is noteworthy that an inverse correlation between chlorogenic acid content and α-glucosidase inhibition was found, suggesting again that other compounds, such as Maillard reaction products, besides phenolic ones may play a role in inhibiting the enzyme.

As *in vitro* digestion induced changes in some chemical and functional properties of coffee brew, the particle size distribution was also investigated (Figure 15). The undigested coffee brew presented a multimodal particle size distribution, revealing the presence of two

families of compounds, with an average diameter corresponding to 255 ± 10 and 5560 ± 16 nm, respectively.

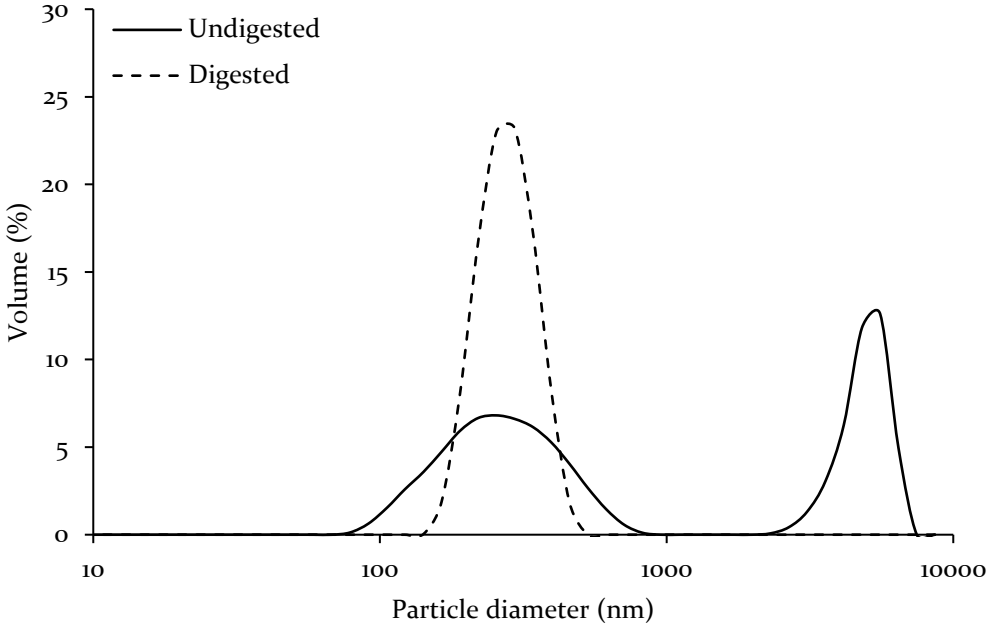


Figure 15. Particle size distribution of coffee brew, before and after *in vitro* digestion.

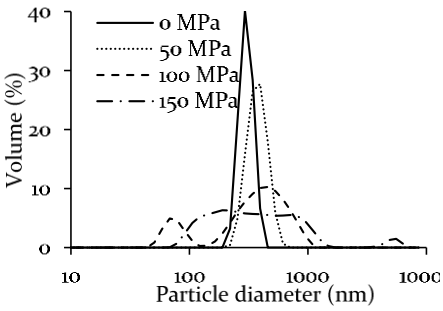
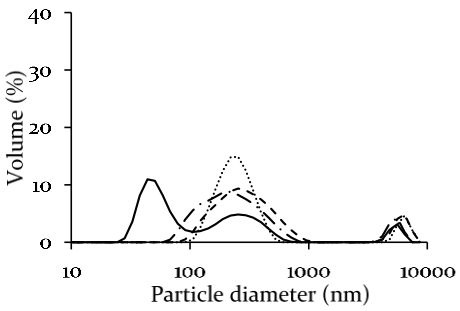
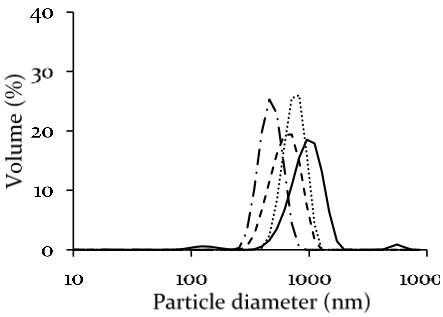
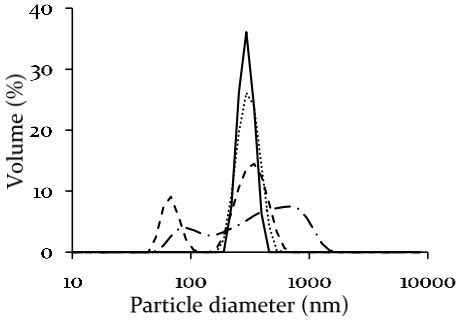
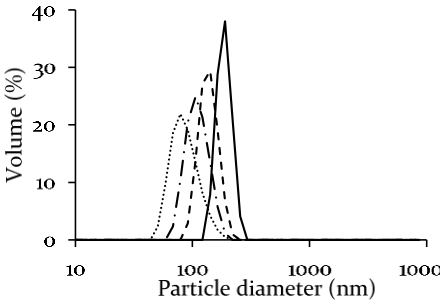
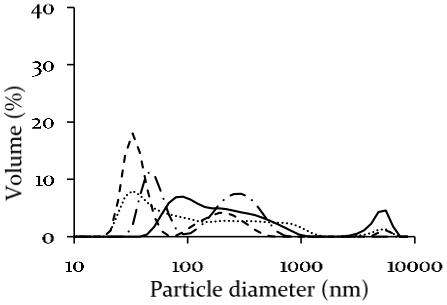
The smaller family could be represented by phenolic compound-protein aggregates, while larger particles could have formed during coffee powder production, upon water evaporation, which is able to induce the irreversible aggregation of coffee components (Gmoser *et al.*, 2017; Le Bourvellec & Renard, 2012). Upon *in vitro* digestion, a single family of compounds (298 ± 5 nm) was observed. The latter might have formed as a result of the digestive enzyme activity, leading to the degradation of native coffee brew particles and the formation of new ones. In particular, the presence of bile salts, together with amphiphilic molecules deriving from coffee (i.e. fatty acids and phospholipids), could lead to the formation of mixed micelles, containing several digestion products (Salvia-Trujillo *et al.*, 2017).

A significant change ($p < 0.05$) in the ζ-potential was also observed upon *in vitro* digestion. In particular, non-digested coffee presented a value of -17.45 ± 1.34 mV, while the ζ-potential of digested sample was -32.45 ± 1.63 mV. Such a difference can be attributed to the presence of bile salts and other digestion products on mixed micelle surface, leading to higher stability of the digested system, due to particle repulsion (Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013).

3.3.3.2 Effect of the addition of milk on coffee properties upon *in vitro* digestion

As coffee is often consumed with the addition of milk and this was demonstrated to affect the bioaccessibility of phenolic compounds (Tagliazucchi *et al.*, 2012), in the second part of this research the effect of milk addition, and relevant fat content, and particle size distribution on coffee inhibitory capacity against α-glucosidase was evaluated. In particular, coffee was added with milk (1:1 w/w) having a different fat concentration (0.1, 3.6 and 7.1%), homogenized with high pressures (50, 100 and 150 MPa) and *in vitro* digested. Table 17 shows the particle size distribution of coffee beverages before and after *in vitro* digestion.

Table 17. Particle size distribution of coffee beverages containing 0.1%, 3.6%, and 7.1% fat, before and after *in vitro* digestion.

Fat content (%)	Before in vitro digestion	After in vitro digestion
0.1		
3.6		
7.1		

Increasing fat concentration to 7.1% produced an overall reduction in the average particle diameter of coffee beverages before *in vitro* digestion. HPH also affected the particle size distribution. When considering the lowest fat concentration (<0.1%), peaks can be mainly related to milk protein aggregates that, upon HPH treatment, were disrupted as evidenced by the change from monomodal to multimodal particle distribution. On the contrary, in beverages containing milk at 3.6 and 7.1% fat, the observed signals can be regarded to both fat globules and proteins aggregates. After HPH treatments the monomodal distribution was maintained with a significant shift to lower particle sizes. In particular, by increasing the pressure, a reduction in average particle diameter from 955 to 396 nm was noted for the 3.6% fat containing beverage and from 190 nm to 106 nm in the case of that with 7.1% fat. As expected, the mechanical forces suffered by the product during HPH process induced the disruption of dispersed particles, giving a reason for emulsions with a different structural organization.

After *in vitro* digestion, three particle families were identified: the first ranging from 30 to 70 nm, the second from 220 to 300 nm and the last around 5500 nm. Particles with an average diameter of 295 nm were also observed in digested coffee brew (Figure 15) and were attributed to the presence of mixed micelles. The occurrence of smaller and larger particles upon digestion of coffee beverages could instead be attributed to the presence of milk. Several authors (Garcia, Antona, Robert, Lopez, & Armand, 2014; Hayes & Kelly, 2003; Mercan, Sert, & Akın, 2018) observed two major particle families, with average diameter in the order of magnitude of 100 and 10,000 nm, respectively, upon homogenization and *in vitro* digestion of milk. The first family could be represented by fat globules, while the second might include both aggregates of fatty acids, bile salts and undigested fat particles (Salvia-Trujillo, Qian, Martín-Belloso, *et al.*, 2013; H. Singh *et al.*, 2009), as well as digested milk proteins (Koutina, Ioannidi, Melo Nogueira, & Ipsen, 2017). Interestingly, the higher was the fat content, the higher was the volume of smaller particles also after *in vitro* digestion (Table 17). These results suggest that the presence of higher fat concentrations could not only promote the formation of smaller particles upon HPH treatment but also induce the development of smaller micelles during digestion. To this regard, Salvia-Trujillo, Qian, Martín-Belloso, *et al.* (2013) observed that increasing the lipid surface area exposed to pancreatic lipase led to a decrease in droplet size obtained upon digestion and to a higher extent of lipid digestion. To understand if the differences observed in particle size distribution also affected the stability of undigested and digested coffee beverages, the latter were further analyzed for their ζ-potential (Figure 16).

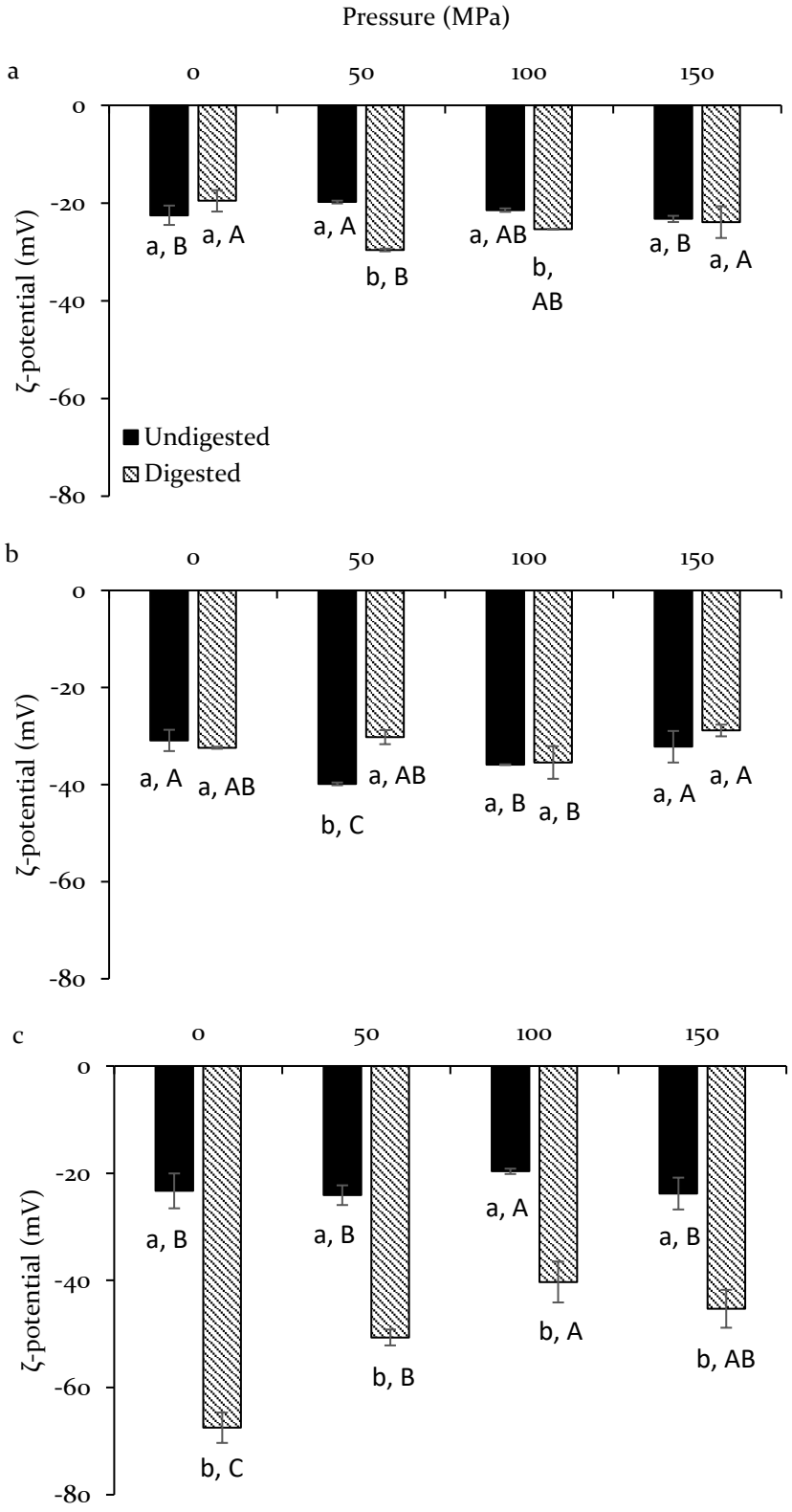


Figure 16. ζ-Potential of coffee beverages containing 0.1% (a), 3.6% (b) and 7.1% (c) of fat and homogenized with different pressure before and after *in vitro* digestion. Lowercase letters (a-b) indicate significant differences before and after *in vitro* digestion, uppercase letters (A-C) indicate significant differences among different pressures within the same sample.

Overall, HPH did not affect the ζ-potential of undigested coffee beverages, while it decreased as fat concentration increased from 0.1 to 3.6% (Figure 16). A further increase to 7.1% of fat did not modify the ζ-potential. *In vitro* digestion only produced minor changes in the ζ-potential when lower fat concentrations were considered. On the contrary, an outstanding difference was observed in the ζ-potential upon *in vitro* digestion of the coffee beverage containing the highest fat concentration, with a decrease ranging from 2- to 3-fold (Figure 16c). It is noteworthy that samples presenting the lowest ζ-potential (Figure 16c), and thus potentially high stability, also presented the smallest particle size distribution upon *in vitro* digestion (Table 17), suggesting that high fat concentration would promote the formation of more stable digesta. To this regard, Otemuyiwa *et al.* (2017) reported that the presence of fat could aid the stabilization of the system during digestion by favoring mixed micelles stabilization and solubilization. These authors also reported that the addition of milk, and thus the presence of fat, plays a crucial role in enhancing phenolic availability of tea and cocoa infusions, by helping their micellarization. On the contrary, other authors reported that the presence of milk may impair phenolic compounds bioaccessibility mainly due to the binding with milk proteins (Duarte & Farah, 2011; Dupas *et al.*, 2006). To get an insight into the effect of the presence of milk with different fat concentration as well as of HPH treatment on chlorogenic acids, the latter were quantified in coffee beverages before and after *in vitro* digestion and their bioaccessibility was computed (Table 18).

Table 18. Bioaccessibility (%) of 3-caffeoylquinic (3-CQA), 4-caffeoylquinic (4-CQA), 5-caffeoylquinic (5-CQA), 5-feruloylquinic (5-FQA) and 4-feruloylquinic (4-FQA) acids, and total chlorogenic acids (Total CGA) in coffee beverages with different fat concentration and homogenized with different pressure. Different letters (a-d) indicate significant differences ($p < 0.05$).

	0.1% fat				3.6% fat				7.1% fat			
CGA	0 MPa	50 MPa	100 MPa	150 MPa	0 MPa	50 MPa	100 MPa	150 MPa	0 MPa	50 MPa	100 MPa	150 MPa
3 CQA	92 ± 1^{ac}	118 ± 8^{ab}	57 ± 5^{c}	80 ± 4^{ac}	98 ± 6^{ac}	95 ± 7^{ac}	127 ± 25^{a}	137 ± 16^{a}	97 ± 5^{ac}	101 ± 2^{ac}	69 ± 2^{bc}	91 ± 4^{ac}
4 CQA	95 ± 22^{ab}	126 ± 4^{a}	59 ± 7^{b}	80 ± 3^{ab}	100 ± 6^{ab}	95 ± 19^{ab}	118 ± 25^{ab}	112 ± 1^{ab}	88 ± 2^{ab}	91 ± 2^{ab}	65 ± 1^{b}	86 ± 2^{ab}
5 CQA	90 ± 14^{ab}	104 ± 4^{a}	45 ± 5^{b}	64 ± 1^{ab}	73 ± 5^{ab}	72 ± 18^{ab}	90 ± 19^{a}	76 ± 12^{ab}	72 ± 3^{ab}	69 ± 1^{ab}	47 ± 1^{b}	68 ± 3^{ab}
5 FQA	96 ± 9^{bc}	155 ± 1^{a}	61 ± 5^{c}	73 ± 11^{bc}	84 ± 5^{bc}	83 ± 15^{bc}	104 ± 18^{b}	83 ± 13^{bc}	92 ± 17^{bc}	103 ± 2^{bc}	76 ± 2^{bc}	102 ± 3^{bc}
4 FQA	105 ± 19^{ac}	105 ± 5^{ac}	59 ± 15^{bcd}	61 ± 9^{bcd}	80 ± 4^{ad}	82 ± 11^{ad}	119 ± 18^{a}	109 ± 29^{ab}	50 ± 3^{d}	52 ± 2^{cd}	38 ± 1^{d}	53 ± 1^{cd}
Total CGAs	93 ± 7^{ab}	112 ± 1^{a}	51 ± 2^{b}	69 ± 1^{ab}	81 ± 5^{ab}	81 ± 18^{ab}	102 ± 19^{a}	93 ± 1^{ab}	77 ± 4^{ab}	78 ± 1^{ab}	54 ± 2^{b}	74 ± 3^{ab}

As shown in Table 18, the bioaccessibility of chlorogenic acids in coffee beverages was strongly affected by the presence of milk. In all cases, the addition of milk increased dramatically the bioaccessibility of CGA moving from about 20-25% (Table 16) to values higher than 50%, up to more than 100%. These results highlighted the reduced susceptibility of CGA to degradation upon *in vitro* digestion, as compared to coffee without milk, in which up to 80% of CGA was lost after digestion (Table 16). To this regard, Duarte & Farah (2011) reported that the effect of milk on CGA content upon digestion would depend on the milk to coffee ratio, and thus on the fat to coffee ratio, which can result in a positive or negative effect on CGA bioaccessibility. In our experimental condition, the 1:1 ratio between milk and coffee may considerably improve the health-promoting performances of coffee. Moreover, the bioaccessibility was shown to be affected both by fat content and HPH intensity. Overall, an increase in phenolic bioaccessibility was observed when coffee beverages contained milk with 0.1% fat and were treated at 50 MPa, as well as when 3.6% fat was considered, and the beverage was treated at 100 MPa. This pressure, however, when combined with the lowest (i.e. 0.1%) and the highest fat level (i.e. 7.1%) led to a significant decrease in chlorogenic acid content, which accounted for nearly 50% of total CGA. Different factors could concomitantly affect the CGA bioaccessibility. As already pointed out, the presence of fat could reduce the susceptibility of CGA to degradation by promoting their micellarization (Otemuyiwa *et al.*, 2017). In addition, phenolic compounds may complex with milk proteins and thus result less prone to breakdown during digestion (Dupas *et al.*, 2006; Lamothe, Azimy, Bazinet, Couillard, & Britten, 2014). Finally, the system particle size could modulate the digestion kinetics and thus CGA bioaccessibility (Salvia-Trujillo, Qian, Martín-Belloso, *et al.*, 2013).

Although contradictory effects were reported in the literature regarding the simultaneous consumption of milk with phenolic compounds on their bioaccessibility (Duarte & Farah, 2011; Tagliazucchi *et al.*, 2012), this controversy was explained by Del Rio, Borges, and Crozier (2010) considering the different concentration of phenolic compounds. According to these authors, milk could interfere with the absorption in the case of low phenolic concentration, but it could have an opposite effect if the phenolic concentration is high enough, as in the case of coffee brew (Del Rio *et al.*, 2010).

Since the addition of milk to coffee brew induced changes in the physical and chemical properties upon *in vitro* digestion, digested coffee beverages, as well as digested milk samples, were further analyzed for the inhibition against α-glucosidase (Figure 17).

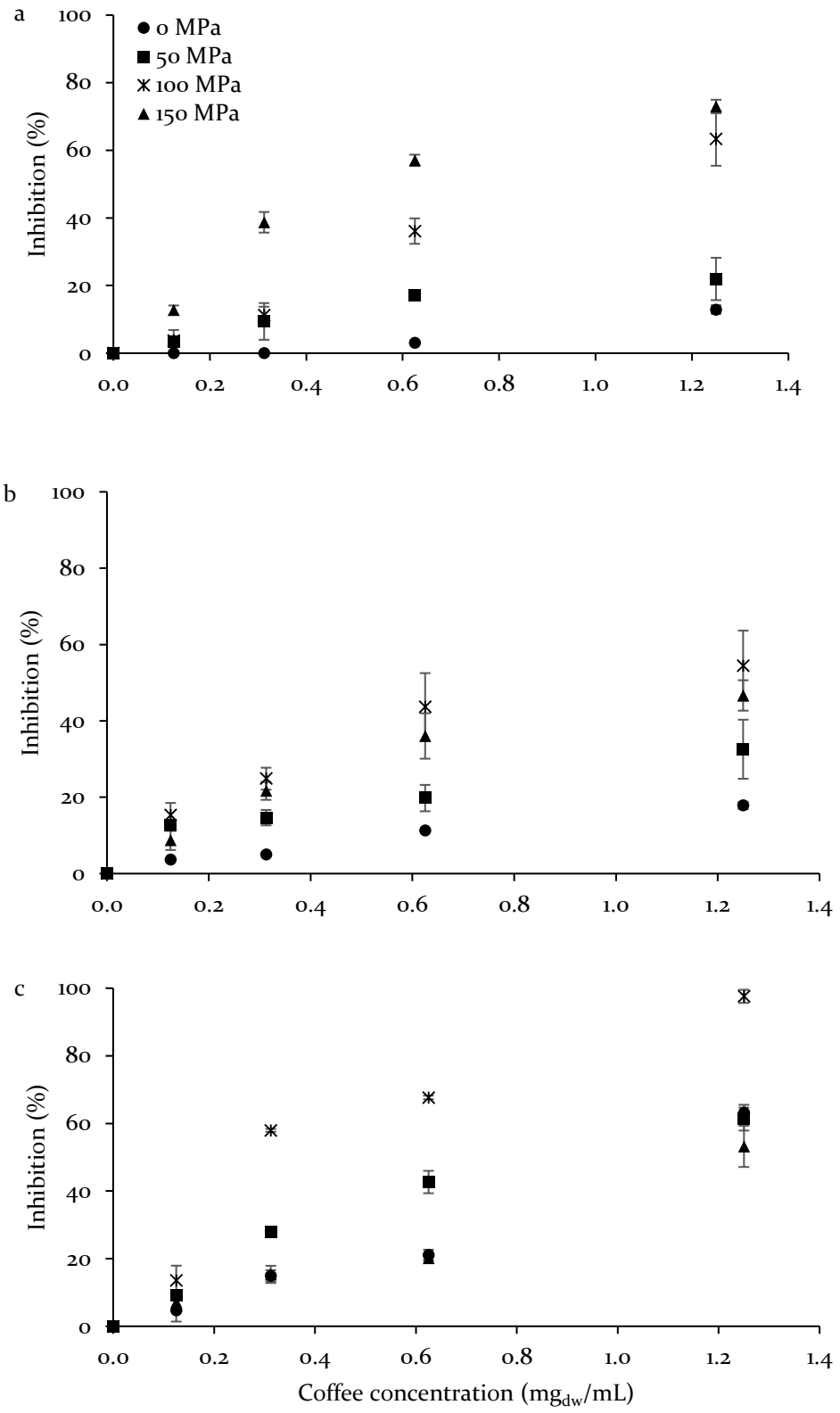


Figure 17. α-Glucosidase inhibitory activity of digested coffee beverages containing 0.1% (a), 3.6% (b) and 7.1% (c) of fat and homogenized with different pressure as a function of coffee concentration.

Digested milk did not inhibit the enzyme in the whole range of analyzed concentrations (data not shown), while digested coffee beverages inhibited α-glucosidase in a concentration-dependent manner (Figure 17). The highest efficacy was observed for the beverage containing milk with 7.1% fat and treated at 100 MPa. Overall, the use of higher pressures (i.e. 100 and 150 MPa) improved the α-glucosidase inhibitory capacity of all beverages. The fat content also affected the ability to inhibit the enzyme. It can be noticed that when 3.6% fat was used, all coffee beverages showed a lower inhibitory capacity against α-glucosidase as compared to coffee brew (Figure 14). On the contrary, lower (0.1%) or higher (7.1%) fat levels improved the ability of coffee beverages to inhibit the enzyme. To our knowledge, despite some information is present in the literature regarding the effect of milk addition on the bioaccessibility of coffee phenolic compounds (Tagliazucchi *et al.*, 2012), for the first time, these data demonstrated that milk addition may affect coffee ability to inhibit α-glucosidase.

3.3.3.3 Identification of the best fat-pressure combination able to minimize IC_{50}

To define the best performing process conditions to obtain a coffee beverage with improved α-glucosidase inhibitory capacity, a 2-factors face-centered central composite design (CCF) was used. To this aim, fat concentration and pressure were considered as independent variables and their effect on IC_{50} was studied (Table 15). The regression coefficients and the relative analysis of variance of the polynomial models for the dependent variable, namely IC_{50}, were computed (Table 19).

Table 19. Regression coefficients of the models for IC_{50} of digested coffee beverages.

Variable	IC_{50}
Intercept	1.014 ***
Fat concentration	-0.581 **
Fat concentration^2	-0.55 *
Pressure	-0.860 ***
Pressure^2	1.299 ***
Fat concentration × Pressure	1.185 ***
R^2_{adj}	0.950

(*) p<0.05, (**) p<0.01, (***) p<0.001

Finally, to evaluate the effect of the independent variables on the dependent one and to predict the optimum values of each variable for minimum IC_{50} to be achieved, a contour-plot was generated (Figure 18).

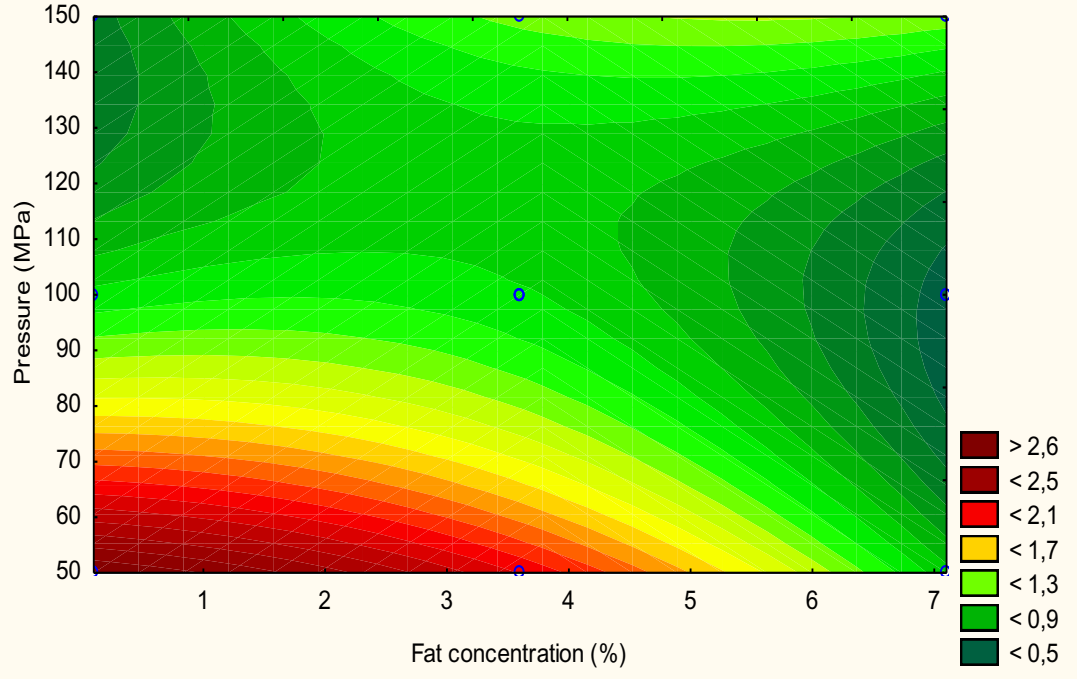


Figure 18. Fitted contour plot of IC50 as a function of fat concentration in coffee beverages and homogenization pressure.

As reported in Figure 18, the IC_{50} could be minimized by different combinations of fat concentration and pressure. In particular, when a low lipid concentration was used (<0.5%), higher pressures (>125 MPa) would be required to minimize the IC_{50}. On the other hand, when a higher lipid concentration was used (around 7%), the IC_{50} could be minimized by applying intermediate pressures (between 80 and 110 MPa). When these conditions were applied, an IC_{50} lower than 0.5 mg_{dw}/mL was obtained. This means that a proper combination of fat concentration and pressure could almost double the ability of coffee to inhibit α-glucosidase since the IC_{50} of coffee brew without milk was 0.94 mg_{dw}/mL.

However, it is noteworthy that the combinations able to minimize the IC_{50} led to a significant decrease in CGA bioaccessibility (Table 18). It can be thus inferred that the inhibitory effect against α-glucosidase might not rely on CGA content, despite these were reported to present antidiabetic effects through α-glucosidase inhibition (Ishikawa *et al.*, 2007). As already reported in the previous part relevant to undigested coffee, an inverse relationship was found between the phenolic content and the efficacy in inhibiting α-glucosidase. The latter could be carried out by other compounds formed upon processing, among which are melanoidins that can incorporate phenolic compounds (Perrone *et al.*,

2012). Moreover, it can be inferred that even in the presence of a higher bioaccessibility, complexation phenomena occurring between phenolic compounds and milk proteins upon high-pressure homogenization (Paquin, 1999) and digestion (Hasni *et al.*, 2011) might result in a reduced bioactivity (Serafini *et al.*, 2009).

3.3.4 Conclusions

The results acquired in the present study pointed out that both formulation (i.e. the addition of milk with different fat content) and processing (i.e. homogenization pressure) play a key role in determining the bioaccessibility of coffee CGA. A proper combination of milk and pressure also increased the potential of coffee to reduce type 2 diabetes risk by improving its ability to inhibit α-glucosidase, through IC_{50} minimization. However, this bioactivity was not related to CGA bioaccessibility, and could thus rely on the presence of other compounds, as well as on more complex interactions occurring upon processing and digestion.

Chapter 4 Apple juice

Apples are one of the most commonly consumed fruits worldwide. In 2018 apple production accounted for more than 100 million of tons (USDA, 2018). The consumption of apples is known to reduce the risk of chronic diseases, such as cancer, cardiovascular diseases and type 2 diabetes (Boyer & Liu, 2004; X. Guo, Yang, Tang, Jiang, & Li, 2017).

The famous sentence: "An apple a day keeps the doctor away!" is what is thus highly recommended and heavily advertised nowadays to stay fit and healthy (Francini & Sebastiani, 2013). The health-protective effect of apples and, in particular, their antidiabetic properties have mainly been attributed to polyphenols, and in particular to the chemical families of flavones (e.g. luteolin, apigenin), flavonols (e.g. quercetin, kaempferol), flavanols (e.g. catechin, epicatechin), hydroxycinnamic acids (e.g. chlorogenic acid) and anthocyanidins (Boyer & Liu, 2004; Hanhineva *et al.*, 2010; Shoji *et al.*, 2017). The biological activity of phenolic compounds has been attributed for a long time to their antioxidant properties (Boyer & Liu, 2004; Nicoli *et al.*, 1999; Nicoli, Anese, Parpinel, Franceschi, & Lerici, 1997). However, in recent years several authors debunked the hypothesis of a direct connection between health effects and antioxidant activity, even when the latter is retained upon digestion (Del Rio *et al.*, 2013; Ludwig, Clifford, *et al.*, 2014; Song *et al.*, 2005). Other mechanisms were suggested to explain the health-related properties of phenolic compounds. Apple phytochemicals were recently demonstrated to affect carbohydrate metabolism and glucose homeostasis at different sites (Hanhineva *et al.*, 2010). For instance, individual phenolic compounds (e.g. catechol, catechin, chlorogenic, ferulic and caffeic acid) extracted from apple reduce the intestinal glucose uptake through the inhibition of the membrane transporter SGLT1 (Schulze *et al.*, 2014). These compounds also act by inhibiting α-glucosidase (Agustinah *et al.*, 2016; Tadera, 2006). To this regard, Bortolotto and Piangiolino (2013) reported that an apple extract inhibited the activity of α-glucosidase by 90%. Despite these studies showed the potential of apples in facing the risk of type 2 diabetes, most of them deal with purified extracts, whereas the relationship between the whole fruit intake and the reduced diabetes risk has not been fully elucidated yet.

In addition, apples are not only eaten raw, but they are often consumed as processed products, such as puree, cider, and juice. In fact, apple juice is the most popular apple derivative and around 25% of harvested apples are allocated for juice production (Schulze *et al.*, 2014). Apple juice production implies several technological interventions, among which are skin and pomace removal, enzymatic depectinization, and pasteurization. These technological treatments, which are intended to improve the stability of fruit and vegetable derivatives, are known to affect their physical and chemical properties (Krapfenbauer, Kinner, Gössinger, Schönlechner, & Berghofer, 2006), often impinging the fresh-like features. Thus, they undeniably affect also the phenolic content of the final product (Schulze *et al.*, 2014; Van Buren, De Vos, & Pilnik, 1976) and consequently its potential health benefits. However, no indication regarding the effect of pasteurization on the antidiabetic properties of apple juice, and more specifically on its ability to inhibit α-glucosidase, are currently available in the literature.

In addition, it should be pointed out that during the last decade a large part of food research has been driven by the increasing market demand for ready-to-eat and minimally processed vegetable derivatives (Rocha & Morais, 2007). Unconventional technologies, based on thermal or non-thermal processes, have been proposed to prevent processed food from losing its freshness (González-Aguilar, Ayala-Zavala, Olivas, de la Rosa, & Álvarez-Parrilla, 2010; Rastogi, 2011; Rico, Martín-Diana, Barat, & Barry-Ryan, 2007). This currently represent one of the most important aspects affecting consumer choice (Sillani & Nassivera, 2015), as they are increasingly aware of the potential health benefits deriving from the consumption of vegetable products (Carbonell-Capella *et al.*, 2014). Thus, the need to identify processing conditions able not only to preserve the sensory characteristics but also to maintain or even improve food nutritional properties is crucial to meet consumer expectations.

Extended literature review has already been provided about the different impact of conventional and unconventional technologies on the phenolic content of several vegetable matrices (Rico *et al.*, 2007; Soliva-Fortuny & Martín-Belloso, 2003). However, investigating the effect of technological interventions on phenolic content is not exhaustive when studying health-related properties, as it is necessary to take into account also the effect of digestion (Ferruzzi, 2010). Still, most studies related to the antidiabetic properties, and more specifically to the α-glucosidase inhibitory capacity, of apple juice have been carried out on bioactive compounds extracted from apples (Williamson, 2013), thus neglecting the effect of processing and digestion.

4.1 Effect of thermal pasteurization on *in vitro* α-glucosidase inhibitory activity of apple juice, and study of the interaction of apple juice with acarbose *in vitro* and in healthy rats

4.1.1 Aim of the study

The aim of the present study was to investigate the effect of thermal pasteurization on the *in vitro* inhibitory activity of apple juice against α-glucosidase. Further, for the first time the interaction between apple juice and acarbose was studied, to understand whether their combination might allow drug dosage reduction while keeping the efficacy against α-glucosidase. The combination study was carried out both *in vitro* and *in vivo* on healthy rats. Part of the results here presented has been published:

- ✓ Alongi, M., Verardo, G., Gorassini, A., Anese, M. (2018). Effect of pasteurization on *in vitro* α-glucosidase inhibitory activity of apple juice. *LWT - Food Science and Technology, 98*, 366-371.

4.1.2 Material and methods

4.1.2.1 Materials

Methanol (MeOH), formic acid (HCOOH), fructose, glucose, sucrose, (+)-catechin, (–)-epicatechin, chlorogenic acid, phloridzin, phloretin, 3-hydroxycinnamic acid (internal standard; I.S.), α-glucosidase, 4-nitrophenyl-α-D-glucopyranoside, and acarbose were purchased from Sigma-Aldrich (Milan, Italy). Quercetin-3-*O*-galactoside, procyanidin B2, and epigallocatechin gallate were obtained from ExtraSynthese (Lyon, France). Quercetin-3-*O*-arabinoside and quercetin-3-*O*-rhamnoside were purchased from Carbosynth (Berkshire, UK). Milli-Q grade water was produced by Elgastat UHQ-PS system (ELGA, High Wycombe Bucks, UK).

Solid phase extraction (SPE) columns ISOLUTE C18, 1 g, 6 mL were from Biotage (Milan, Italy).

4.1.2.2 Sample preparation

A 10 kg batch of apples (*Malus domestica Borkh.*, cv. Golden Delicious) was purchased at the local market and maintained at 7 °C until use. Apples were washed, wiped and the juice was extracted (Ariston Hotpoint Slow Juicer, Fabriano, Italy) at 4 °C to minimize enzymatic browning. The juice was centrifuged at 5000 ×*g* for 5 min at 4 °C (Beckman Avanti J-25 Beckman Instruments Inc., Palo Alto, CA, USA) and filtered through filter paper. Approximately 1.5 kg of apples was used for each replicate. Ten mL aliquots of apple juice were poured into 20 mL capacity glass vials (Vetrotecnica, Padova, Italy), which were closed with screw caps and kept refrigerated. Samples were subjected to technological treatments within 10 min after preparation.
Commercial apple juice (Mela limpida 100%, Skipper-Zuegg, Verona, Italy) was also purchased on the local market.

4.1.2.3 Pasteurization

Thermal treatments were performed in a silicone oil bath (Haake Phoenix B5, Thermo Electron Co., Karlsruhe, Germany). Samples were pasteurized by applying two different time-temperature combinations. A copper-constantan thermocouple probe (Ellab, Denmark), whose tip (2.0 mm) was placed in the coldest point of the sample (i.e. at two-thirds of depth in glass vials), measured temperature changes of apple juice during pasteurization. The thermal effect *F* (min) was computed using Equation 8 (Ball, 1923):

$$F = \int_0^t 10^{(T-T_{ref})/z} \cdot dt \qquad \text{Equation 8}$$

where T_{ref} is the reference temperature, *T* is the actual temperature of the treatment (°C), *t* is the time (min). The first treatment ($P_{71.7}$) provided a sterilizing equivalent to 0.4 min at 71.7 °C and aimed at achieving 5 Log reductions of *Cryptosporidium parvum* ($D_{71.7}$=3 s and z=5 °C) (FDA, 2003). The second treatment (P_{90}) provided a sterilizing equivalent to 14.8 min at 90 °C and aimed at reducing by 2 Log *Alicyclobacillus acidoterrestris* (D_{90}=7.4 min and z=12 °C) (Silva & Gibbs, 2001). After treatment, the samples were rapidly cooled in a spray of water until they reached a temperature of approximately 30 °C. Apple juice not subjected to heat treatment was taken as a control.

4.1.2.4 Total solid content and pH

The total solid content was measured by a gravimetric method (AOAC, 1995). pH was measured by a pHmeter (HANNA Instruments, pH 301, Padova, Italy).

4.1.2.5 Sugar content

The method by Englyst, Englyst, Hudson, Cole, & Cummings (1999) was followed, upon slight changes. Apple juice was mixed with methanol (1:5, mL: mL), left at room temperature for 1 h and centrifuged at 4000 ×*g* for 10 min at 4 °C (Beckman Avanti tm J-25, Beckman Instruments Inc., Palo Alto, CA, USA). The supernatant was analyzed using an HPLC pump (LC-10AT VP, Shimadzu, Japan) equipped with a refractive index detector (RID-10A, Shimadzu, Japan). An inverse phase apolar C18 column (5 µm, 250 x 4.6 mm) was used (Grace Davison Discovery Sciences, Alltima, Lokeren Belgium). The injection valve (Rheodyne, Sigma-Aldrich, Milano, Italia) was equipped with a 20 µL plastic loop and samples were injected using a syringe (SGE LC, 100 µL, FN). The mobile phase was represented by acetonitrile and deionized water (70:30, mL: mL) and 1.3 mL/min flow rate was applied. Quantitative analysis of sugars was carried out by comparing the sugar peak area with the results of calibration lines obtained by injecting fructose, glucose, and sucrose standard solutions serially diluted. Calibration lines were linear (R^2 > 0.995) in the 1.0 to 250.0 g/L concentration interval.

4.1.2.6 Phenolic composition

SPE purification

Ten μL 3-hydroxycinnamic acid (50 μg/mL) methanolic solution as internal standard and 1 mL juice was diluted with 2 mL deionized water and loaded on a C18 SPE column previously conditioned with 5 mL of 2 mL/L formic acid in methanol and 5 mL of 20 mL/L formic acid in water.

After loading, the column was washed with 10 mL of 20 mL/L formic acid in water and the phenolic fraction was eluted with 5 mL methanol. The solvent was removed, and the residue was properly diluted with H_2O/MeOH (9:1, mL: mL). The solution was transferred to an autosampler vial for the HPLC-DAD-ESI-MS/MS analysis.

HPLC-DAD-ESI-MS^n analysis

The method by Kahle, Kraus, & Richling (2005) was followed, with slight changes. Chromatographic analysis was performed with a Dionex Ultimate 3000 UPLC (Thermo Scientific, San Jose, CA, USA) equipped with a thermostated autosampler and a column oven. The UPLC system was coupled with a diode array detector and an electrospray ionization mass detector (HPLC-DAD-ESI-MS^n) in parallel by splitting the mobile phase 1:1. Negative-ion ESI mass spectra were obtained with a Finnigan LXQ linear trap mass spectrometer (Thermo Scientific, San Jose, CA, USA). The typical ESI source conditions were transfer line capillary at 275 °C; ion spray voltage at 3.30 kV; sheath, auxiliary and sweep gas (N_2) flow rates at 10, 5 and 0 arbitrary units, respectively. Helium was used as the collision damping gas in the ion trap set at a pressure of 0.13 Pa. ESI-MS^n spectra were obtained by collision-induced dissociation (CID) experiments after isolation of the appropriate precursor ions in the ion trap (isolation width 1.2 *m/z* unit), and subjecting them to the following typical conditions: normalized collision energy between 20% and 30%, selected to preserve a signal of the precursor ion in the order of 5%; 0.25 activation Q and 30 ms activation time.

The chromatographic separation was performed with a column Synergi Hydro, 4 mm, 250 x 2.0 mm (Phenomenex, Italy), thermostated at 30 °C. Elution was carried out at 0.3 mL/min flow rate, using as mobile phase 2 mL/L formic acid in methanol (A) and 2 mL/L formic acid in water (B) with the following gradient: 0-6 min 10% A, 20 min 40% A, 40 min 40% A, 46 min 100% A, 52 min 100% A, 54 min 10% A, 54-60 min 10% A. The injection volume was 20 µL. The acquisition was carried out in full scan (*m/z* 50 - 1500) and in full scan MS^2 (*m/z* 50 - 600) selecting the precursor ion $[M-H]^-$ at *m/z* 289.1 for (+)-catechin and (-)-epicatechin, *m/z* 577.2 for procyanidin B2, *m/z* 457.1 for epigallocatechin gallate, *m/z* 353.1 for chlorogenic acid, *m/z* 163.0 for 3-hydroxycinnamic acid (I.S.), *m/z* 463.1 for quercetin galactoside, *m/z* 435.2 for phloridzin, *m/z* 433.1 for quercetin xyloside and quercetin arabinoside, *m/z* 447.1 for quercetin rhamnoside and *m/z* 273.1 for phloretin, respectively. Phloretin-xyloglucoside ($[M-H]^-$ *m/z* 567.2; MS^2: *m/z* 273) and 4-*p*-coumaroylquinic acid ($[M-H]^-$ *m/z* 337.1; MS^2: *m/z* 173, 163, 155) were tentatively characterized, by comparison of their fragmentation pattern with those available in the literature (Sommella *et al.*, 2015).

The quantitative analysis was carried out using an Ultimate 3000 RS Diode Array detector (Thermo Scientific, San Jose, CA, USA) controlled by Chromeleon software (version 6.80). Spectral data from all peaks were accumulated in the range 200-400 nm and chromatograms were recorded at 280 nm for (+)-catechin, (-)-epicatechin, procyanidin B2, epigallocatechin

gallate, 3-hydroxycinnamic acid (I.S.), phloretin-xiloglucoside, phloridzin, phloretin, 314 nm for 4-*p*-coumaroylquinic acid, 328 nm for chlorogenic acid, 258 nm for quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside and quercetin-3-*O*-rhamnoside, respectively.

Calibration curves and quantification

A stock solution of (+)-catechin, (–)-epicatechin, procyanidin B2, epigallocatechin gallate, chlorogenic acid, phloridzin, phloretin, quercetin-3-*O*-galactoside, quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinoside, quercetin-3-*O*-rhamnoside in H_2O/MeOH (9:1, mL: mL) was serially diluted with the same solvent to prepare 7-point calibration curves in the range 12-3000 ng/mL with a constant concentration of the I.S. (500 ng/mL). The R^2 coefficients for the calibration curves were > 0.99. When standards were unavailable, the quantification of the analyte was carried out using the calibration curve of available standard presenting similar chemical structure.

4.1.2.7 Color analysis

Color analysis was carried out as described in paragraph 3.1.2.4.

4.1.2.8 α-Glucosidase inhibition assay

The *α*-glucosidase inhibitory activity was determined spectrophotometrically (UV-2501PC, UV-Vis recording Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) as previously described in paragraph 3.1.2.6. Apple juice was tested in the concentration range 0-23 mg_{dw}/mL.

4.1.2.9 Study of apple juice and acarbose interaction *in vitro*

The method proposed by Chou and Talalay (1984) was used, with some modifications, to investigate apple juice and acarbose interaction towards *α*-glucosidase inhibition. The inhibitory activity (%) against *α*-glucosidase, namely effect *x*, carried out by $P_{71.7}$ apple juice or acarbose was represented as a function of apple juice or acarbose concentration, respectively.

Combined systems of juice and acarbose with a proportionally increasing concentration of both juice and acarbose were obtained according to Equation 9:

$$Fm_n \times \left[(D_m)_j + (D_m)_a\right] = (C_n)_{j,a}$$ Equation 9

where Fm_n represents a multiplicative factor, $(D_m)_j$ and $(D_m)_a$ are the doses of juice and acarbose able to produce a 50% α-glucosidase inhibition (i.e. IC_{50}) and $(C_n)_{j,a}$ is the total concentration of juice and acarbose in the combined system. Six combined systems were obtained by substituting six different multiplicative factors (i.e. 0.25; 0.5; 1.5; 2; 2.5; 3.5) in Equation 9. The combined systems were tested for their ability to inhibit α-glucosidase and the inhibition percentage was plotted against the concentration. The sum of juice and acarbose doses $(D_x)_{j,a}$ corresponding to an effect x was thus determined, and the relevant single doses of juice and acarbose were calculated by Equation 10 Equation 11, respectively:

$$(D)_j = (D_x)_{j,a} \times \frac{(D_m)_j}{(D_m)_a + (D_m)_j}$$ Equation 10

$$(D)_a = (D_x)_{j,a} \times \frac{(D_m)_a}{(D_m)_a + (D_m)_j}$$ Equation 11

The Combination Index (CI) was finally calculated by Equation 12:

$$CI = \frac{(D)_j}{(D_x)_j} + \frac{(D)_a}{(D_x)_a}$$ Equation 12

where $(D)_j$ represents the dose of juice in the combined system producing an effect x, $(D)_a$ represents the dose of acarbose in the combined system producing the same effect x, $(D_x)_j$, is the dose of juice alone producing the effect x and $(D_x)_a$ is the dose of acarbose alone producing the effect x.

4.1.2.10 Study of apple juice and acarbose interaction in healthy rats

Young adult (six-week-old) male Wistar rats (*Rattus Norvegicus*, n = 20) weighing 242 ± 12 g were obtained from Envigo RMS Srl (San Pietro al Natisone, Italy). They were housed in wire-bottomed cages in a room with controlled temperature (25° C) and lighting (12 h light/dark cycle) and had free access to water and to a commercial diet (Envigo RMS Srl, San Pietro al Natisone, Italy) for 1 week. All procedures were carried out according to the guidelines enforced in Italy (Decreto Legislativo n. 116, 1992) and in compliance with the

guide of the National Research Council (National Research Council, 2011), upon approval by the Italian Committee for Bioethics.

After 15 h fasting, apple juice combined with different dosages of acarbose was orally administered to rats. In particular, aliquots of 3.6 mL juice were administered based on the available carbohydrate content. Considering apple juice sugar concentration, this was equal to 1.58 g/kg_{bw}, which is the concentration recommended to test the glycemic response in rat models (Belobrajdic, Wei, & Bird, 2016). Acarbose dosage corresponded to 32.28 mg/kg_{bw} and was chosen based on the typical human daily dose of acarbose (300 mg/60 kg_{bw}) and according to the conversion factor reported by Huang, Huang, Chen, Zheng and Sun (2004). Similarly to what reported by Q. Zhang *et al.* (2013), two additional dosages were selected, corresponding respectively to 16.14 and 3.23 mg/kg.

Blood samples were collected from tail veins at 0 (prior to the administration), 15, 30, 45, 60, 90 and 120 (after the administration) min to assay plasma glucose concentration (Accu-Chek®, Roche Diabetes Care Italy S.p.A., Monza, Italy) (Belobrajdic *et al.*, 2016), which was plotted against time.

4.1.2.11 Statistical analysis

Results are averages of at least three measurements carried out on two replicated samples and are reported as means ± standard deviation for *in vitro* assays and as means ± standard error for *in vivo* trials. Statistical analysis was performed using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p < 0.05$). Best fitting analysis was carried out using Table Curve 2D (version 4.0, SPSS Inc., Chicago, IL, USA) and the goodness of fitting was evaluated based on statistical parameters of fitting (R^2, p and standard error SE).

4.1.3 Results and discussion

4.1.3.1 Effect of pasteurization on some chemical and physical properties of apple juice

Apple juice with average total solid content and pH of 13.7 ± 2.1 g/100 mL and 3.7 ± 0.1, respectively, were subjected to two different pasteurization processes providing sterilizing

effects equivalent to $F_{71.7}^{5}$=0.4 min and F_{90}^{12}=14.8 min. As expected, total solid content and pH did not change upon heat treatment. Table 20 shows sugar concentration and color parameters of untreated and pasteurized apple juices.

Table 20. Sugar concentration and color parameters of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min (P71.7) or F_{90}^{12}=14.8 min (P90). Means in the same column indicated by a common letter (a-c) are not significantly different (p > 0.05).

Sample	Sucrose (g/L)	Fructose (g/L)	Glucose (g/L)	L*	Hue angle (arctan b*/a*)
Raw	20 ± 12^{ab}	159 ± 35^{ab}	7 ± 1^{a}	51 ± 2^{b}	70 ± 1^{b}
$P_{71.7}$	33 ± 1^{a}	201 ± 1^{a}	5 ± 1^{a}	50 ± 1^{b}	71 ± 1^{b}
P_{90}	8 ± 1^{b}	109 ± 1^{b}	5 ± 1^{a}	59 ± 1^{a}	87 ± 1^{a}

Fructose was the most abundant sugar in apple juice samples, followed by sucrose and glucose. Raw and $P_{71.7}$ samples did not significantly differ for sugar content, while fructose and sucrose in P_{90} sample were approximately 30 and 60% less concentrated than those found in the control. This reduction can be attributed to sugar consumption as reagents of the Maillard reaction. On the contrary, glucose concentration did not change significantly, probably due to the heat-induced release of aglycone compounds, and thus of glucose, that might counterbalance the Maillard reaction effect (Rivas, Rodrigo, Martínez, Barbosa-Cánovas, & Rodrigo, 2006).

Pasteurization processes, especially the most intense one, caused an increase in L* and hue angle values, which are indices of sample bleaching. Such an increase may be due to hydrolysis of polymeric aggregates formed upon polyphenoloxidase activity and consequently to the formation of low molecular weight, soluble compounds (McKenzie & Beveridge, 1988).

4.1.3.2 Effect of pasteurization on α-glucosidase inhibitory activity of apple juice

Figure 19 shows α-glucosidase inhibitory activity as a function of the total solid concentration of untreated apple juice.

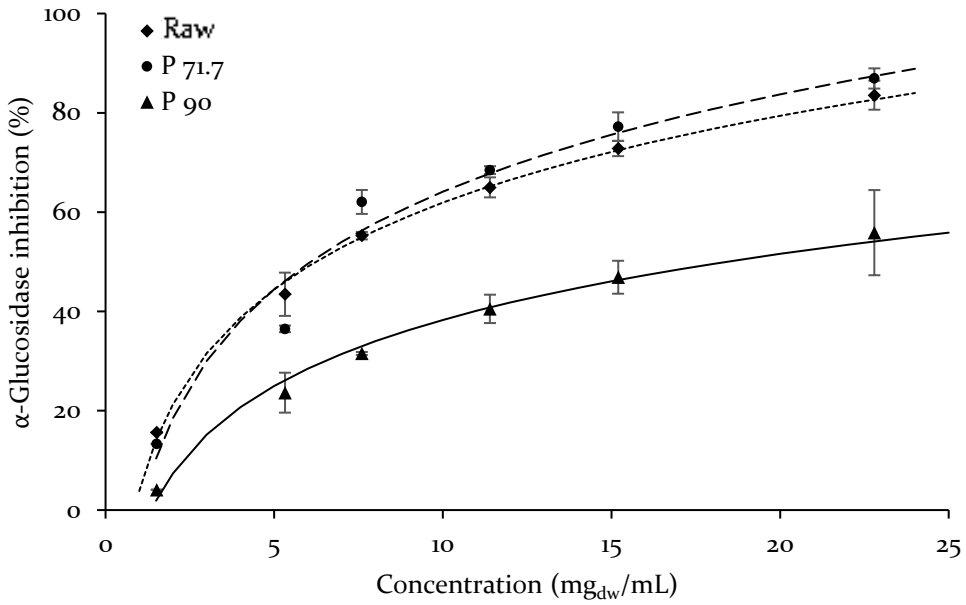


Figure 19. α-Glucosidase inhibitory activity of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min ($P_{71.7}$) and F_{90}^{12}=14.8 min (P_{90}), as a function of total solid concentration. Data fitting: lines, estimates; symbols, experimental data.

Apple juice significantly inhibited the enzyme activity in a dose-dependent way, in agreement with literature data (Adyanthaya, Kwon, Apostolidis, & Shetty, 2010; Ankolekar *et al.*, 2012; He, Yang, Zhang, Ma, & Ma, 2014; Nasu *et al.*, 2005). The enzymatic activity was suppressed by almost 85% when apple juice concentration was 23 mg$_{dw}$/mL.

Figure 19 also shows α-glucosidase inhibitory activities of the pasteurized apple juices as a function of total solid concentration. No significant changes in the α-glucosidase inhibitory activity of the $P_{71.7}$ apple juice were found as respect to the untreated sample. On the contrary, the most intense pasteurization was responsible for approximately 35% decrease in the α-glucosidase inhibitory activity of P_{90} apple juice.

The potential of apple derivatives in reducing the postprandial rise in blood glucose has already been reported (Adyanthaya *et al.*, 2010; Ankolekar *et al.*, 2012; Nasu *et al.*, 2005; Shoji *et al.*, 2017). Several authors (Boyer & Liu, 2004; X. Guo *et al.*, 2017) described an inverse relationship between apple consumption and risk of type 2 diabetes. The α-glucosidase

inhibitory effect carried out by apple juice was attributed to naturally present phenolic compounds (Schmidt, Lauridsen, Dragsted, Nielsen, & Staerk, 2012; Williamson, 2013). Based on these considerations, the latter were quantified in apple juice submitted or not to pasteurization (Table 21), to understand whether thermal processing-induced changes in phenolic compound profile, potentially affecting the α-glucosidase inhibitory capacity of apple juice.

Table 21. Phenolic compound concentrations of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min ($P_{71.7}$ sample) and F_{90}^{12}=14.8 min (P_{90} sample). n.d.: not detectable

	Raw (mg/L)	**$P_{71.7}$ (mg/L)**	**P_{90} (mg/L)**
Chlorogenic acid	1.12 ± 0.02	3.85 ± 0.01	16.26 ± 0.15
p-Coumaroylquinic acid	0.89 ± 0.04	2.28 ± 0.03	4.35 ± 0.14
∑ Hydroxycinnamic acids	**2.01**	**6.13**	**20.61**
Phloretin	n.d.	n.d.	n.d.
Phloretin xyloglucoside	0.27 ± 0.02	0.69 ± 0.01	2.30 ± 0.02
Phloridzin	0.13 ± 0.00	0.38 ± 0.01	1.18 ± 0.01
∑ Dihydrocalcone derivatives	**0.40**	**1.06**	**3.47**
Epigallocatechin gallate	0.21 ± 0.01	0.25 ± 0.01	n.d.
(+) Catechin	n.d.	n.d.	n.d.
Procyanidin B2	n.d.	n.d.	0.20 ± 0.00
(-) Epicatechin	n.d.	0.10 ± 0.01	3.79 ± 0.13
∑ Flavanols	**0.21**	**0.36**	**3.99**
Quercetin-3-*O*-galactoside	0.85 ± 0.02	1.18 ± 0.03	1.42 ± 0.06
Quercetin-3-*O*-hexoside	0.08 ± 0.01	0.87 ± 0.00	0.97 ± 0.04
Quercetin-3-*O*-xyloside	0.12 ± 0.00	0.18 ± 0.00	0.23 ± 0.00
Quercetin-3-*O*-arabinoside	n.d.	n.d.	n.d.
Quercetin-3-*O*-rhamnoside	1.45 ± 0.02	1.55 ± 0.01	1.65 ± 0.02
Others quercetin-pentoside	0.21 ± 0.01	0.28 ± 0.01	0.31 ± 0.01
∑ Flavonols	**2.72**	**4.06**	**4.59**
Total phenolic compounds	**5.33**	**11.61**	**32.66**

As reported in Table 21, the number of phenolic compounds differed in apple juice submitted or not to pasteurization. The total phenolic content increased concomitantly with the intensity of thermal treatment, in agreement with literature findings (Agcam, Akyildiz, Evrendilek, Akyıldız, & Evrendilek, 2014; Gerard & Roberts, 2004). In particular, 2-fold and 6-fold increases in total phenolic content were found when the mildest and most intense pasteurization treatments were applied, respectively. Among phenolic compounds, flavonols and hydroxycinnamic acids represented the major classes, accounting for nearly 90% of the overall content in untreated juice. Chlorogenic acid was the most abundant compound and its concentration markedly increased upon the most intense treatment, accounting for 50% of the total phenolic content. Other authors reported this compound to be the most abundant phenol in apple derivatives and observed an increase in its concentration upon thermal treatment (Keenan, Brunton, Butler, Wouters, & Gormley, 2011).

The increase in phenolic compounds upon pasteurization could be attributed to thermal induced hydrolysis of the most heat-labile compounds (such as epigallocatechin gallate and procyanidin polymers) resulting in the release of monomers and dimers, among which are (-)-epicatechin and procyanidin B2 (De Paepe *et al.*, 2014).

The increase in phenolic compounds can be also caused by thermal inactivation of polyphenoloxidase. This enzyme is well known to induce phenolic compound polymerization in vegetable matrices, producing complex dark-colored pigments (Oszmianski, Wolniak, Wojdyło, & Wawer, 2008). By inactivating polyphenoloxidase, pasteurization can thus prevent phenolic compounds from being involved in browning reactions (Ioannou, Hafsa, Hamdi, Charbonnel, & Ghoul, 2012).

Overall, acquired data indicate that the reduction in the inhibitory activity (Figure 19) corresponded to an increase in total phenolic content (Table 21). On the contrary, Adyanthaya *et al.* (2010) found that an increase or decrease in α-glucosidase inhibition corresponded to a similar rise and fall in the concentration of phenolic compounds. However, it is noteworthy that literature data refer to unprocessed juice. The results of the present study suggest the existence of more complex mechanisms underlying α-glucosidase inhibition. In particular, the decrease in the inhibitory activity could be attributed to the thermally induced degradation of bioactive molecules other than phenolic compounds (Shori, 2015).

4.1.3.3 Apple juice-acarbose interactive capability of inhibiting the α-glucosidase activity

The α-glucosidase inhibitory capacity of apple juice was compared to that carried out by acarbose (Table 22).

Table 22. IC_{50} (half-maximal inhibitory concentration) and acarbose equivalents of apple juice not pasteurized (Raw) or subjected to pasteurization providing sterilizing effects equivalent to $F_{71.7}^{5}$=0.4 min ($P_{71.7}$ sample) and F_{90}^{12}=14.8 min (P_{90} sample).

Inhibitor	IC_{50} (mg/mL)	Acarbose equivalents ($IC_{50\ acarbose}/IC_{50\ inhibitor}$)
Raw	6.24	0.014
$P_{71.7}$	6.08	0.014
P_{90}	18.41	0.004
Acarbose	0.09	1

Table 22 reports the IC_{50} of untreated, $P_{71.7}$, P_{90} apple juices, and acarbose solution, as well as the acarbose equivalents of the enzyme inhibitors.

As expected, acarbose was more effective in inhibiting α-glucosidase than apple juice (Table 22). Raw and $P_{71.7}$ apple juices were 100-fold less effective than acarbose in inhibiting the enzyme. However, considering their dry matter (137 mg/mL) and the suggested daily intake of acarbose (300 mg/d), 220 mL/d of juice would provide an acarbose like effect. This dose should be increased to 547 mL/d when the most intensely treated juice (P_{90}) is considered since the latter was 250-fold less effective than acarbose in inhibiting α-glucosidase. These results demonstrated the efficacy of apple juice in inhibiting α-glucosidase *in vitro*, considering ordinarily consumed amounts of juice, as reported in the literature (Nasu *et al.*, 2005).

The combined system of apple juice and acarbose was also analyzed, to understand if apple juice may play a role in enhancing the inhibitory effect carried out by acarbose. The interaction of acarbose with conventionally pasteurized apple juice (i.e. $P_{71.7}$) was investigated. Figure 20 shows the α-glucosidase inhibition produced by apple juice, acarbose and apple juice-acarbose combined system.

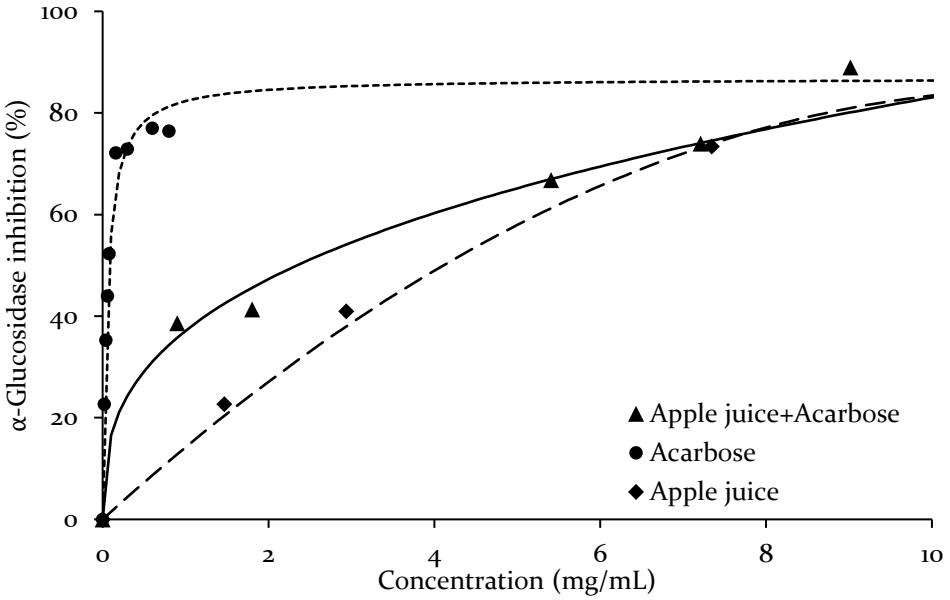


Figure 20. α-Glucosidase inhibitory activity of apple juice, acarbose and the apple juice-acarbose combined system as a function of total solid concentration. Data fitting: lines, estimates; symbols, experimental data.

It can be noticed that the combined system of apple juice and acarbose was more effective than apple juice alone in inhibiting the enzyme. Better to understand the interactive behavior of apple juice and acarbose, the combination index (CI) was computed and plotted as a function of the inhibition percentage against α-glucosidase (Figure 21).

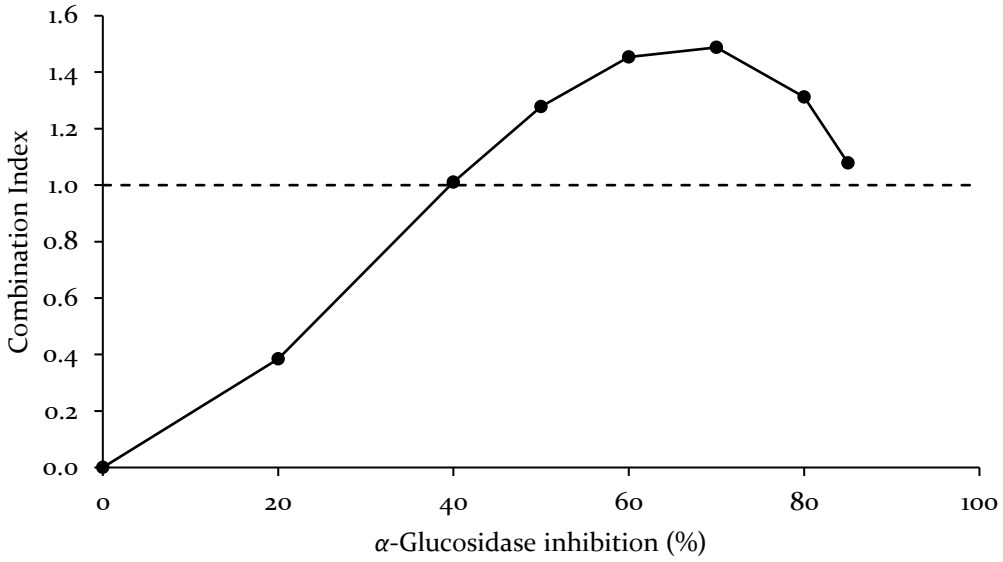


Figure 21. Combination index (CI) relevant to apple juice-acarbose combined system as a function of the α-glucosidase inhibition percentage. CI > 1: antagonistic effect; CI < 1: synergic effect; CI = 1: additive effect.

To this purpose, six combined systems were prepared by multiplying six multiplicative factors (i.e. 0.25; 0.5; 1.5; 2; 2.5; 3.5) to the IC_{50} (i.e. the concentration required to cause 50% α-glucosidase inhibition) of apple juice and acarbose. As already mentioned, the CI provides an indication of the interaction between apple juice and acarbose in the overall enzyme inhibition range. In particular, CI > 1 indicates antagonistic effect; CI < 1 means synergic effect and CI = 1 stands for additive effect. As reported in Figure 21, apple juice and acarbose played a synergistic effect up to 40% inhibition, which corresponded to a concentration of apple juice-acarbose combined system near to 2 mg/mL (Figure 20). An antagonistic behavior between apple juice and acarbose was found when the inhibition was between 40 and 80%, corresponding to a concentration of the combined system ranging from 2 to 9 mg/mL. However, it is noteworthy that, when inhibition percentages were higher than 70%, the CI progressively decreased, approximating the additive effect when 90% inhibition was approached.

As different combinations of acarbose and apple juice differently affected the inhibitory activity against α-glucosidase, thus potentially modifying the glycemic response and paving the way for drug dosage reduction, further research considering *in vivo* trials is ongoing. In particular, rats were administered with commercial apple juice combined with three different dosages of acarbose and the glycemic response was measured. Preliminary result are shown in Figure 22.

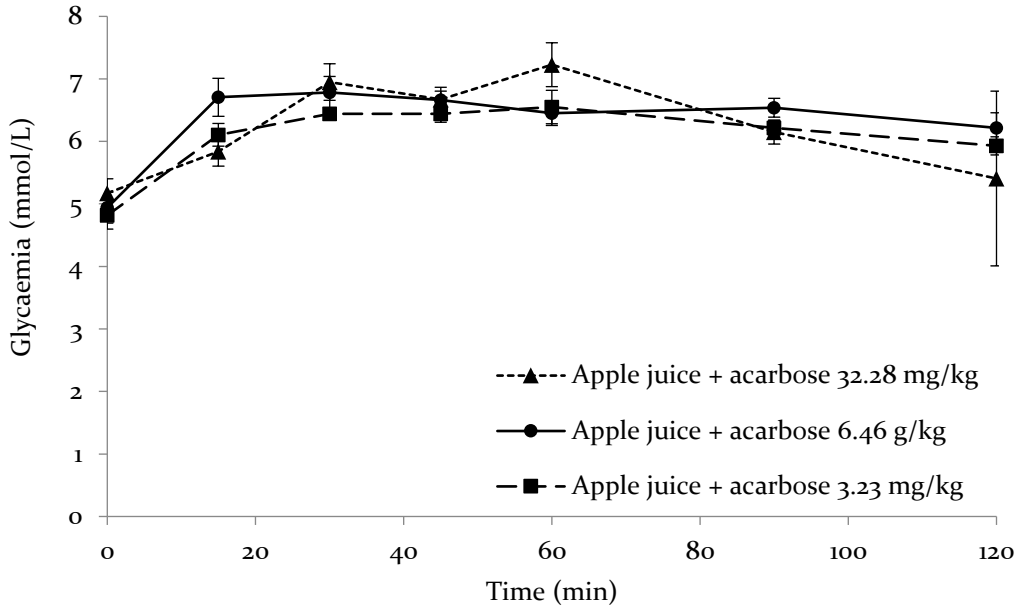


Figure 22. Glycemic response of apple juice combined with different dosages of acarbose.

Data obtained through the *in vivo* experiment partially substantiated results acquired by investigating *in vitro* the interactive behavior of juice and acarbose towards α-glucosidase. In fact, when apple juice was administered in combination with acarbose, a decrease and a flattening in the glycemic response was observed as acarbose dosage was reduced. In other words, increasing the concentration of acarbose seemed to reduce its efficacy when combined with apple juice, suggesting that the observation window of the *in vivo* trial lied within the antagonistic range observed during the *in vitro* test (Figure 21). Nevertheless, results pointed out that combining acarbose with apple juice allowed reducing its dose while increasing the antidiabetic efficacy, thus potentially representing a dietetic approach to limit drug side effects. Still, it should be kept in mind that studying this interaction by *in vitro* and *in vivo* trials provides different outcomes that hardly can be matched in a straightforward manner. Further research is thus required to understand the mechanism behind the interactive behavior between apple juice and acarbose, to identify the combination leading to a synergistic effect also *in vivo*.

4.1.4 Conclusions

The results of the present study draw attention to the functional properties of apple juice, in terms of phenolic content and antidiabetic potential. Acquired data demonstrated that milder pasteurizationdid not significantly affect the physical and chemical properties of apple juice, nor its inhibiting ability against α-glucosidase. By contrast, severe pasteurization caused a prominent increase in phenolic content, but negatively affected the antidiabetic capacity of apple juice. This suggests that carrying out a technological intervention required to previously define the targeted functionality, which relies upon an adequate choice of processing parameters. To do this, it is essential to consider how digestion affects food matrix composition and bioactivity. Moreover, results showed that the apple juice-acarbose combined system played a synergistic effect up to 40% α-glucosidase inhibition, whereas higher concentrations led to an antagonistic behavior. These observations were partially confirmed by *in vivo* trials. Obtained results may represent a starting point to further investigate the potential effect of apple juice in bolstering the efficacy of the drugs conventionally used for the treatment of type 2 diabetes. Nonetheless, it should be considered that apple juice is an important source of sugars. The latter should be taken into account when designing foods aimed at reducing type 2 diabetes incidence. Thus, further studies may consider apple juice derivatives with low sugar concentration to be exploited for their antidiabetic effect.

4.2 Phenolic content and potential bioactivity of apple juice as affected by thermal and ultrasound pasteurization

4.2.1 Aim of the study

The present paper aimed at investigating the effect of conventional (i.e. thermal) and unconventional (i.e. ultrasound) pasteurization of apple juice along with the effect of digestion on the phenolic content and bioaccessibility, the antioxidant activity, the inhibitory capacity against α-glucosidase, and the binding ability to human serum albumin, chosen as a model blood transport protein. Part of the results here presented has been published:

- ✓ Alongi, M., Verardo, G., Gorassini, A., Lemos, M. A., Hungerford G., Cortella, G., & Anese, M. (2019). Phenolic content and potential bioactivity of apple juice as affected by thermal and ultrasound pasteurization. *Food & Function, 10*, 7366-7377.

4.2.2 Material and methods

4.2.2.1 Materials

The same materials as reported in paragraph 4.1.2.1 were used. In addition, acetic acid (CH_3COOH), α-amylase from *Bacillus sp*., porcine pepsin, porcine pancreatin, porcine bile extract, HCl, NaOH, $CaCl_2(H_2O)_2$, Na_2CO_3, $NaHCO_3$, NaCl, KCl, K_2HPO_4, KH_2PO_4, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$, $MgSO_4$, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulfate, $FeCl_3$, 2,4,6-tri(2-pyridyl)-1,3,5-triazine (TPTZ), Trolox, human serum albumin (>99%), Hank's balanced salt solution (HBSS), Dulbecco's Modified Eagle's Medium (DMEM), glucose-free DMEM, Triton-100, as well as, 10 kDa cut off sacks (Avg. flat width 35 mm) were purchased from Sigma-Aldrich (Milan, Italy). Transwell® insert plates (6 well, polyester membrane, 24 mm diameter, pore size 0.4 μm) were purchased from Corning Inc. (Oneonta, AL, USA).

4.2.2.2 Sample preparation

Apple juice was prepared as previously described in paragraph 4.1.2.2.

4.2.2.3 Thermal and ultrasound pasteurization

Thermal pasteurization (P_{90}, hereafter called T) was carried out as described in paragraph 4.1.2.3 and providing a sterilizing effect equivalent to 14.8 min at 90 °C and aimed at reducing by 2 Log *Alicyclobacillus acidoterrestris* (D_{90}=7.4 min and z=12 °C).

Ultrasound pasteurization was carried out according to Saeeduddin *et al.* (2015), which showed a complete inactivation of microbes (total plate count, yeasts, moulds) upon this treatment. Freshly prepared apple juice (200 mL) was poured into a jacketed beaker connected with a thermostat (Thermo Scientific HAAKE PC 200), to maintain the juice temperature at 65 ± 2 °C during the ultrasound treatment. The latter was carried out for 10 min using an ultrasonic transducer (Hielscher ultrasound technology UP400S) equipped with 22 mm probe and working at 20 kHz.

Immediately after treatments, juice samples were cooled in an ice bath and kept refrigerated until further analysis.

4.2.2.4 Temperature measurement

The temperature was recorded as a function of time using a copper-constantan thermocouple probe (Ellab, Denmark), connected to a data logger (CHY 502A1, Tersid, Milano, Italy).

4.2.2.5 Power and energy density computation

Instantaneous power density values (P_v, W/m^3) during the thermal treatment were estimated based on the temperature increase of the sample based on Equation 13.

$$P_v(T) = \rho c_p(\frac{\partial T}{\partial t}) \qquad \text{Equation 13}$$

were ρ is the juice density (1040 kg m^{-3}), c_p is its heat capacity (3.86 kJ kg^{-1}K^{-1}), T is the temperature (K) and t (s) is time. Adiabaticity was assumed for the vessel, given that a possible increase in the power density due to heat loss would not affect the thermal treatment, because temperature would remain constant.

During the ultrasound pasteurization, significant power density was applied on the sample, which led to a major temperature increase thus requiring the use of a cooling device to control the juice temperature. Due to cooling, it was not possible to estimate the power

density based on the juice temperature values. The ultrasound power density was thus determined in a separate test performed at 65 °C, by recording the initial temperature (T, K) increase against time (t, s) and calculating its initial derivative at quasi-adiabatic conditions (Raso, Mañas, Pagán, & Sala, 1999).
In all cases, the energy density was then estimated by integration according to Equation 14 on the whole treatment time:

$$E_v = \int P_v\,(T)dt \qquad \text{Equation 14}$$

4.2.2.6 Electrical energy consumption

The measurement of electrical energy consumption was performed as reported by Bot *et al.* (2017). The energy requirement was estimated by measuring the electrical consumption at the mains supply. Both the ultrasonic processor and the heater for the thermal treatment were supplied with single-phase 230 V electrical power, and a power meter (PC-300, Lafayette, Taiwan) was connected to measure their electrical power and thus calculate the electrical energy density (MJ/m^3) for the whole treatment. The electrical energy use values must be considered typical of our laboratory apparatus. The cooling phase for both treatments was not taken into account, nor was the cooling applied during US treatment, as it could be performed with a variety of systems (*e.g.* chilled water, tap water, dedicated refrigeration system, ice bath).

4.2.2.7 Total solid content and pH

The total solid content and pH were measured as reported in paragraph 4.1.2.4.

4.2.2.8 Colour analysis

Color analysis was carried out as described in paragraph 3.1.2.4.

4.2.2.9 *In vitro* digestion

In vitro digestion was carried out according to the protocol proposed by Minekus *et al.* (2014), as described in detail in paragraph 2.1.2.9. except for some changes. In particular, pancreatin was used instead of lipase during the intestinal phase. Additionally, at the end of the intestinal phase, samples were poured into 12 kDa cut-off sacks and phosphate buffer solution (0.1 M, pH 7) was added to achieve a ratio of sample to buffer of 1:6.25 (v/v). Dialysis

was carried out at 37 °C for 4 h under stirring and the dialyzed sample was considered as the bioaccessible fraction.

4.2.2.10 Caco-2 cell absorption test

Human colon adenocarcinoma (Caco-2) cells were seeded in 6 well insert plates. After reaching confluency, Caco-2 cells were cultured in DMEM for 21 days to obtain highly differentiated cell monolayers. Before the experiment, these were incubated at 37 °C in glucose-free DMEM for 1 h, washed twice and balanced with HBSS for 30 min.
The bioaccessible fraction resulting from digestion was diluted with pH 5.5 PBS (1:2, v/v), to prevent the cytotoxic effect observed in preliminary trials. An aliquot of 1.5 mL was loaded in the apical compartment, while pH 7.4 PBS (2.0 mL) was placed in the basolateral compartment. After 1 h incubation at 37 °C, the apical and basolateral compartments were collected (Soler-Rivas, Ramirez-Anguiano, Reglero, & Santoyo, 2009). Cell membranes were lysed with 2 mL HBSS containing 1% Triton-100, centrifuged at 14,000 rpm for 15 min at 4 °C and the supernatant was collected (K. Li, Yao, Du, Deng, & Li, 2018).

4.2.2.11 Antioxidant activity

The antioxidant capacity was measured based on the ABTS assay (Miller, Rice-Evan, Davies, Gopinathan, & Milner, 1993). Briefly, 2.5 mL of ABTS 7 mM was added with 44 µL of 140 mM potassium persulfate and 1 mL of this solution was mixed with 0.1 mL sample. The absorbance at 734 nm was recorded after 90 s using an UV-VIS spectrophotometer (GENESYS™ 10S UV-Vis Spectrophotometer, ThermoFisher Scientific).
The FRAP (ferric reducing antioxidant power) assay was also used to measure the antioxidant capacity (Benzie & Strain, 1996). 100 mL of acetate buffer (pH 3.6), 12 mL of distilled water, 10 mL of $FeCl_3$ 20 mM and 10 mL of TPTZ (10 mM) were mixed, and 3 ml of this solution was mixed with 0.9 mL of sample. The absorption at 593 nm was recorded after 4 min. Appropriate solvent blanks were also run. A standard curve (R^2 = 0.99) was plotted with different concentrations of Trolox (0-125 µM) and the antioxidant activity was expressed as Trolox equivalent antioxidant capacity (TEAC).

4.2.2.12 Phenolic compound bioaccessibility and bioavailability

Phenolic compounds in the apple juice samples were analysed before and after *in vitro* digestion as described in paragraph 4.1.2.6. Bioaccessibility was computed as described in

paragraph 2.1.2.12. Bioavailability was expressed as the ratio between the concentration of the compound in the sample collected from the basolateral compartment and that in the digested sample.

4.2.2.13 α-Glucosidase inhibition assay

The inhibitory activity of digested juice samples was assessed as previously described in paragraph 3.1.2.6.

4.2.2.14 Fluorescence measurements

The time-resolved fluorescence measurements were performed using a HORIBA Scientific DeltaFlex fluorescence lifetime system in a "T" format, similar to that described by Hungerford, Lemos, and Chu (2019) for monitoring kinetics at two emission wavelengths simultaneously. Excitation was made using DeltaDiode DD-375L laser and detection was made using HPPD-860 and HPPD-890 hybrid detectors. The very low deadtime "FiPho" timing electronics were run in "photon streaming" mode in order to collect the kinetic binding data. These data were binned to produce histograms every 5 ms. For these studies the emission was monitored at both 415 nm and 510 nm simultaneously. 250 µL of (15 mg/mL) human serum albumin was added to 3mL of US pasteurized and digested sample under stirring (300 rpm) at room temperature. All data were collected and analysed using EzTime software, which enabled the direct calculation of decay associated spectra from the global analysis of the time-resolved emission spectral measurements. Decays were reconvoluted with the instrumental response function (IRF) and fitted to a sum of exponentials. Excitation-emission matrices (EEM's) were collected on a HORIBA Scientific FluoroLog 3.

4.2.2.15 Statistical analysis

Results are averages of at least three measurements carried out on two replicated samples and are reported as means ± standard deviation. Statistical analysis was performed using R (version 3.2.3, The R Foundation for Statistical Computing, Vienna, Austria). Bartlett's test was used to check the homogeneity of variance, one-way ANOVA was carried out and Tukey test was used to determine statistically significant differences among means ($p < 0.05$).

4.2.3 Results and discussion

4.2.3.1 Effect of thermal and ultrasound pasteurization on some physical and chemical properties of apple juice

Raw apple juice presented an average total solid content of 11.6 ± 0.1% and pH = 3.7 ± 0.1. As expected, and pointed out previously (paragraph 4.1.3.1), no changes in these parameters were observed upon thermal and ultrasound pasteurization. By contrast, the different treatments led to significant changes in juice colour (Table 23).

Table 23. Colour parameters of apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization. Different letters (a-c) mean significant differences ($p < 0.05$) among samples.

Sample	L^*	Hue angle (arctan b*/a*)
Raw	29.1 ± 0.5^c	30.8 ± 1.2^c
T	35.5 ± 0.2^a	81.4 ± 0.4^a
US	30.3 ± 0.1^b	68.2 ± 0.5^b

As already observed (Table 20) L* and hue angle values significantly increased upon thermal pasteurization, indicating sample bleaching. When ultrasound pasteurization was applied, the reduced heat intensity allowed minimizing colour changes. The latter represents one of the most important intrinsic attributes determining consumer choice of vegetable derivatives (Abid *et al.*, 2015; Sillani & Nassivera, 2015). Thus, by reducing changes in the appearance of apple juice, ultrasound pasteurization could positively affect the perceived quality of pasteurized apple juice as compared to the thermal one.

4.2.3.2 Effect of thermal and ultrasound pasteurization on the phenolic content of apple juice before and after *in vitro* digestion

As ultrasound pasteurization improved the fresh-like appearance of apple juice (Table 23), further research aimed at understanding its effect, as compared to the thermal pasteurization, on the potential functionality of apple juice. The latter is well known to contain considerable number of phenolic compounds, which have been demonstrated to provide beneficial effects toward health (Boyer & Liu, 2004). Phenolic compounds were thus

analysed in raw, and in thermal and ultrasound pasteurized apple juice (Figure 23), to investigate the effect of different technological interventions on their concentration.

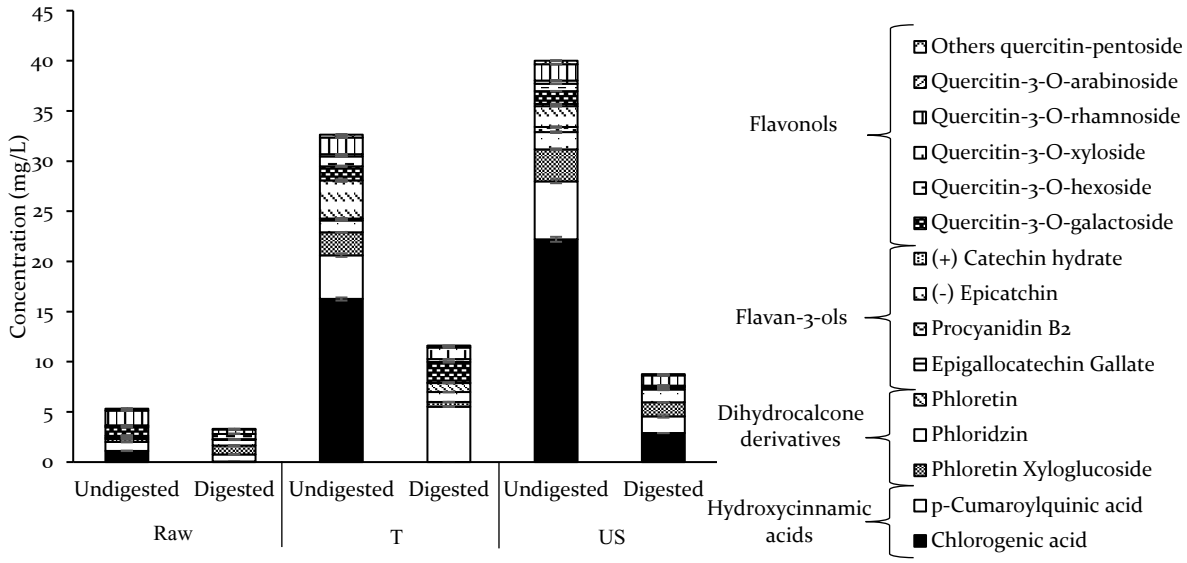


Figure 23. Phenolic compound concentrations of apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization, before and after *in vitro* digestion.

As previously reported (Table 21), the total phenolic concentration increased from 5.33 to 32.66 mg/L when raw apple juice was submitted to thermal pasteurization. The concentration of phenolic compounds in the undigested Raw and T juices, already shown in Table 21, is here reported to ease the comparison with the digested samples.

This relates to polyphenol oxidase inactivation, as well as to the release of monomers and dimers upon thermally induced hydrolysis of heat-labile compounds (De Paepe *et al.*, 2014; Ioannou *et al.*, 2012). When ultrasound pasteurization was used instead, the total phenolic content further increased to 40.03 mg/L, accounting for a 7.5-fold higher concentration than in the Raw apple juice. Despite the lower temperature used during ultrasound pasteurization as compared to the thermal one, the combination of cavitation with heat could have induced tissue disruption, producing not only bacterial and enzymatic inactivation, but also promoting the release of phenolic compounds from the vegetable matrix (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Donnell, Tiwari, Bourke, & Cullen, 2010).

The effect of the treatments was also studied by applying the steady state fluorescence emission through an excitation-emission matrix (EEM). These are shown in Figure 24 for different treatments.

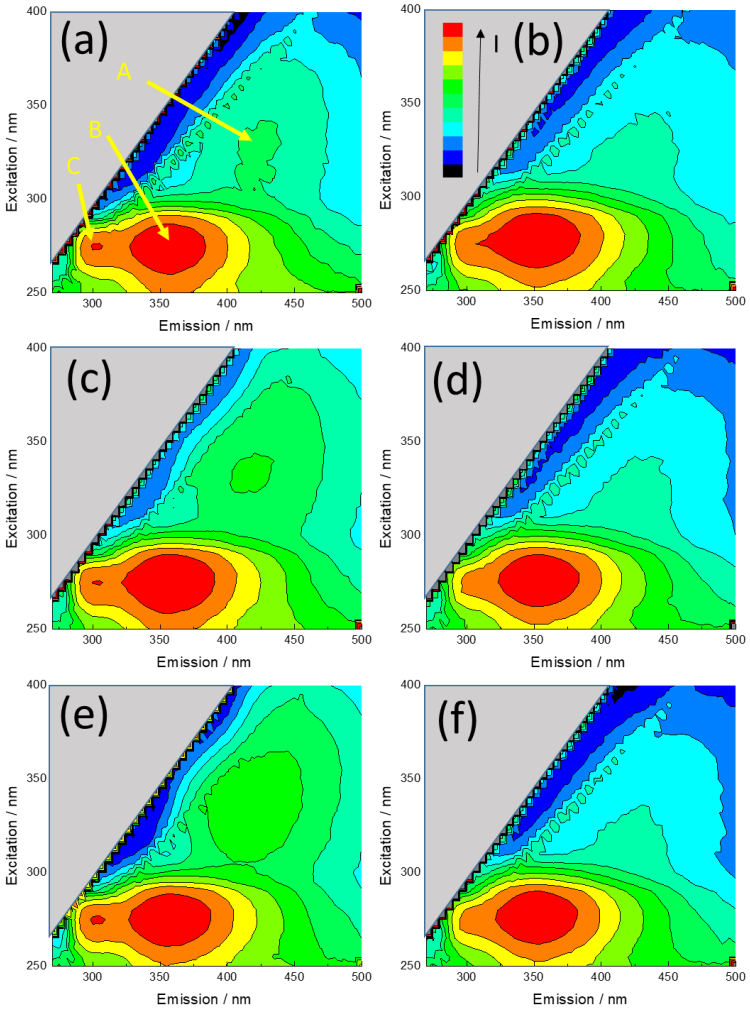


Figure 24. EEMs for (a) raw, (b) raw digested, (c) thermal, (d) thermal digested, (e) ultrasound and (f) ultrasound digested apple juice. The increasing intensity scale (blue to red, indicated in (b)) is logarithmic to clarify lower intensity emissions.

The EEMs shown in Figure 24 are in keeping with those reported (using a larger wavelength range) by Włodarska, Pawlak-Lemańska, Khmelinskii and Sikorska (2016). Here differences in three spectral regions (A, B & C in Figure 24a) can be seen between the undigested and digested samples, as well as depending on the treatment. In the undigested samples, spectral regions C and B which can be attributed in part to emissions from tyrosine and flavanols (Włodarska *et al.*, 2016), are the dominant emissions and appear to be consistent for the raw and treated samples. The main spectral difference between the undigested samples is region A, which has principally been attributed to chlorogenic acid (CGA) (Włodarska *et al.*, 2016). The relative intensity of the emission in this region appears to match changes in the concentration of this compound shown in Table 1, with the highest emission in the US treated sample, followed by the thermal treated and then the raw sample. Upon digestion the greatest change is noted in region A, with the reduction in its relative emission, plus also

a relative reduction is also noted in region C. Again, the result is in keeping with what reported in Figure 23.

When considering different components of the total phenolic compounds in raw apple juice, flavonols and hydroxycinnamic acids represent 51% and 38%, respectively. Even if an overall increase in the concentration was observed for both classes upon pasteurization, flavonols only accounted for 14%, while hydroxycinnamic acids represented the 63% of total phenolic compounds in thermal pasteurized apple juice. Such differences could be attributed to differences in degradation and formation pathways among apple juice phenolic subclasses (De Paepe *et al.*, 2014). The switch in the relative abundance of flavonols and hydroxycinnamic acids was even more pronounced when ultrasound pasteurization was applied, accounting for 10 and 70%, respectively. Although changes were observed in the composition of the phenolic pool upon thermal and ultrasound pasteurization, the latter induced a greater increase in the overall content of phenolic compounds in apple juice. To understand if the increase in phenolic compounds could actually result in an improved functionality of apple juice, the US treated sample was *in vitro* digested. The phenolic compounds were then quantified for the digested samples (Figure 23) and the results obtained were used to compute their bioaccessibility (Table 24).

Table 24. Bioaccessibility of phenolic compounds in apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization. n.d.: not determined.

	Raw (%)	**T (%)**	**US (%)**
Chlorogenic acid	5.07 ± 0.43	0.00 ± 0.00	13.02 ± 0.00
p-Coumaroylquinic acid	78.96 ± 5.32	126.91 ± 0.28	28.61 ± 2.05
Hydroxycinnamic acids	**37.80 ± 2.59**	**26.78 ± 0.06**	**16.24 ± 0.43**
Phloretin Xyloglucoside	333.03 ± 9.13	20.90 ± 0.26	44.17 ± 0.46
Phloridzin	430.32 ± 23.24	82.79 ± 2.08	74.45 ± 3.21
Phloretin	n.d.	n.d.	n.d.
Dihydrocalcone derivatives	**365.50 ± 13.84**	**68.33 ± 1.84**	**54.74 ± 0.82**
Epigallocatechin Gallate	14.36 ± 1.71	n.d.	n.d.
Procyanidin B2	n.d.	0.00 ± 0.00	0.00 ± 0.00
(-) Epicatechin	n.d.	0.49 ± 0.03	0.00 ± 0.00
(+) Catechin	n.d.	n.d.	0.00 ± 0.00
Flavan-3-ols	**14.36 ± 1.71**	**0.47 ± 0.03**	**0.00 ± 0.00**
Quercetin-3-*O*-galactoside	2.58 ± 0.39	145.29 ± 4.41	15.81 ± 1.76
Quercetin-3-*O*-hexoside	654.87 ± 18.40	29.50 ± 0.92	19.64 ± 0.79
Quercetin-3-*O*-xyloside	0.00 ± 0.00	510.92 ± 21.78	16.80 ± 2.70
Quercetin-3-*O*-rhamnoside	29.65 ± 3.41	8.47 ± 0.30	62.59 ± 0.55
Quercetin-3-*O*-arabinoside	n.d.	n.d.	n.d.
Others quercetin-pentoside	32.54 ± 0.97	0.00 ± 0.00	33.90 ± 1.44
Flavonols	**38.78 ± 2.32**	**80.08 ± 2.80**	**35.67 ± 0.69**
Total phenolic compounds	**62.06 ± 3.27**	**35.47 ± 0.56**	**21.91 ± 0.27**

A decrease in total phenolic concentration was observed after *in vitro* digestion of all samples (Figure 23). Such a decrease was more pronounced in the order Raw<T<US, accounting for a 40, 65 and 78% decrease respectively. These results indicate a higher susceptibility of the phenolic compounds to degradation during the digestion process after exposure to technological interventions, resulting in a reduced bioaccessibility (Table 24). Since phenolic compounds were more abundant in pasteurized samples as compared to the raw juice (Figure 23), it can be inferred that they could be more easily available as a substrate for esterase activity at intestinal level (Andreasen, Kroon, & Williamson, 2001). This can be

followed by oxidative degradation, polymerization and complexation with metal ions, proteins and fibres (Rodríguez-Roque *et al.*, 2014), resulting in a more pronounced depletion.

As already pointed out for the different pasteurization processes, besides producing an overall decrease in phenolic concentration, digestion also resulted in a prominent modification in their content profile. After digestion of raw apple juice, flavonols decreased from 51% to 32% of the total phenolic compounds and hydroxycinnamic acids from 38% to 23%, while dihydrochalcones increased from 7 to 44%.

In particular, chlorogenic acid represented the compound most susceptible to degradation, accounting for a 95% reduction in its concentration after digestion of raw apple juice. Such a decrease was even more marked when considering thermally pasteurized apple juice, in which chlorogenic acid, representing alone the 50% of the total phenolic content, completely disappeared upon digestion. Ultrasound pasteurization also produced an important decrease (i.e. 87%) of chlorogenic acid upon digestion (Figure 23). Overall, despite some exceptions (e.g. phloretin and quercetin-3-O-arabinoside only formed upon thermal pasteurization and digestion), carrying out the *in vitro* digestion after pasteurization produced a similar effect on all phenolic compounds, i.e. a more pronounced decrease in their concentration, resulting in a reduced bioaccessibility (Table 24).

Nonetheless, it is important to highlight that upon *in vitro* digestion phenolic concentration in both thermal and ultrasound pasteurized apple juices was still higher as compared to the digested raw sample (Figure 23). In other words, the more pronounced loss of phenolic compounds observed upon digestion in pasteurized samples, as compared to the raw juice, was counterbalanced by the increase in their concentration produced during pasteurization. This resulted in an overall increase in the number of phenolic compounds potentially available for intestinal absorption when pasteurized juices were considered.

Further trials were carried out to assess the bioavailability (BAV) of phenolic compounds upon absorption through Caco-2 cell monolayer from the apical side, i.e. the intestinal mucosa side, to the basolateral chamber, i.e. the side connected with the bloodstream, thus mimicking the intestinal absorption. Preliminary results relevant to the raw apple juice showed that TPC presented a BAV of about 12%. However, only a few compounds, namely *p*-cumaroylquinic acid, phloretin xyloglucoside, and quercitin-3-*O*-hexoside, were found in the basolateral chamber, in which the compounds that crossed the cell layer from the intestinal mucosa to the bloodstream can be found. It is noteworthy that, based on their concentration (Table 24), these compounds were the most abundant of their category in the

digested sample, suggesting that epithelial transport is concentration-driven. To this regard, it is noteworthy that phenolic compounds are mainly absorbed by paracellular diffusion (Konishi & Kobayashi, 2004). In addition, none of the flavan-3-ols was detected in the basolateral side, suggesting that their metabolism mainly occurs at gastrointestinal level. However, further investigation is required to elucidate these results.

4.2.3.3 Effect of thermal and ultrasound pasteurization on the antioxidant capacity of apple juice before and after *in vitro* digestion

Since changes in the concentration and bioaccessibility of phenolic compounds were observed in apple juices submitted to different pasteurization processes (Figure 23 and Table 24), samples were analysed for their antioxidant capacity (Figure 25).

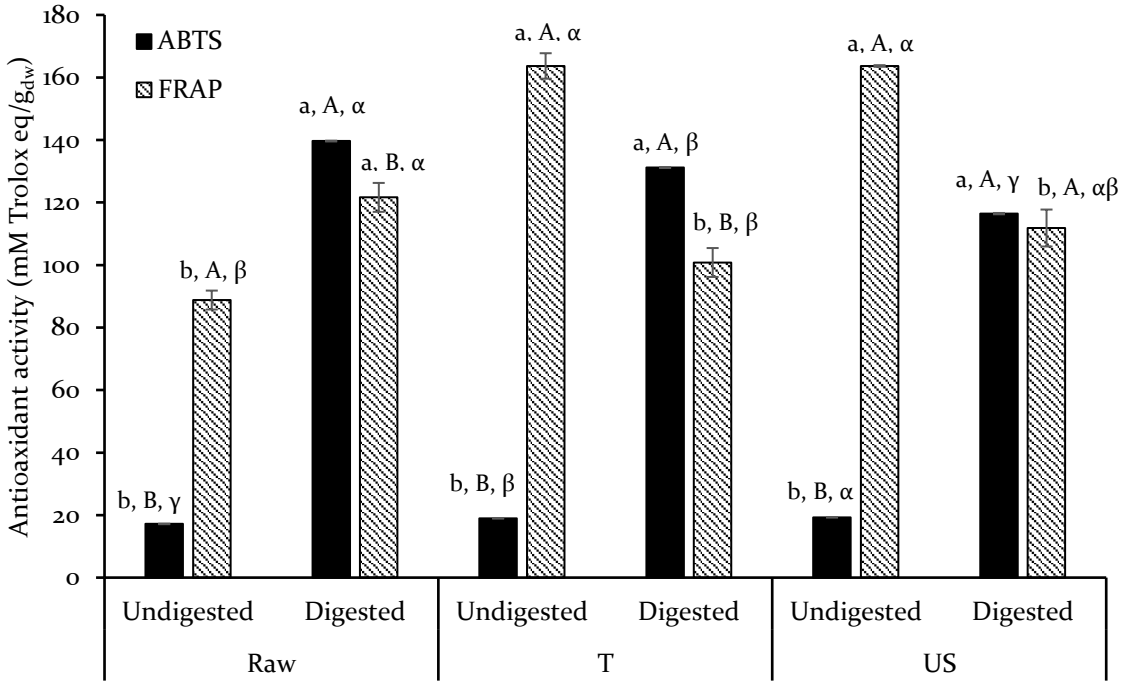


Figure 25. Antioxidant capacity (ABTS and FRAP) of apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization, before and after *in vitro* digestion. Different lowercase latin letters (a-b) indicate significant differences between Undigested and Digested juice subjected to the same treatment (Raw, T, US) and assayed with the same method (FRAP, ABTS). Different uppercase latin letters (A-B) indicate significant differences between FRAP and ABTS assay within the same sample. Different lowercase greek letters (*α-γ*) indicate significant differences among undigested or digested Raw, and T and US treated juices, assayed with the same method (FRAP, ABTS).

As reported in Figure 25, the antioxidant capacity measured by the ABTS assay increased by more than 6-fold after digestion, independently from the application of a pasteurization process, with the highest value corresponding to the raw digested juice. An increase in the

antioxidant capacity upon digestion was also observed by other authors on other phenolic-rich juices (Fawole & Opara, 2016). These results diverge from those relevant to the phenolic content (Figure 23), showing a decrease upon digestion in all cases and thus corroborating the lack of a direct relationship between the health effects attributed to phenolic compounds and the antioxidant capacity (Del Rio *et al.*, 2013; Ludwig, Clifford, *et al.*, 2014). Overall, the results obtained with the FRAP assay significantly differed from those acquired by using the ABTS. In particular, after *in vitro* digestion the antioxidant capacity of thermal (T) or ultrasound (US) pasteurized apple juice decreased when compared to that observed for undigested samples. The more pronounced differences were observed in the undigested samples, while digested ones presented closer antioxidant capacity values. Still, no relation was found between such capacity (Figure 25) and the phenolic content (Figure 23). The different results obtained from the two methods can rely on the different conditions underlying antioxidant capacity measurement, including the oxidant/reductant used as well as the compositional and environmental variables (Nicoli *et al.*, 1999). In particular, the ABTS assay is based on the reduction of the antioxidant due to the loss of an electron by the nitrogen atom of ABTS. On the contrary, the FRAP method relies on the oxidation of the antioxidant leading to the reduction of the complex ferric ion-TPTZ (Pisoschi & Negulescu, 2012). As the response of antioxidants to different conditions differs, it is necessary to apply more than one technique to measure the antioxidant capacity of a complex system, in order to avoid result misinterpretation (Prior *et al.*, 2005). Nonetheless, the antioxidant capacity tested by *in vitro* assays does not provide biologically relevant information on antioxidants as it does not consider their behaviour under physiological conditions (Fawole & Opara, 2016; Ludwig, Clifford, *et al.*, 201).

4.2.3.4 Effect of thermal and ultrasound pasteurization on the α-glucosidase inhibitory capacity of digested apple juice

To understand if and how the different pasteurization processes affected the antidiabetic potential of apple juice, digested samples were analysed for their ability to inhibit α-glucosidase (Figure 26).

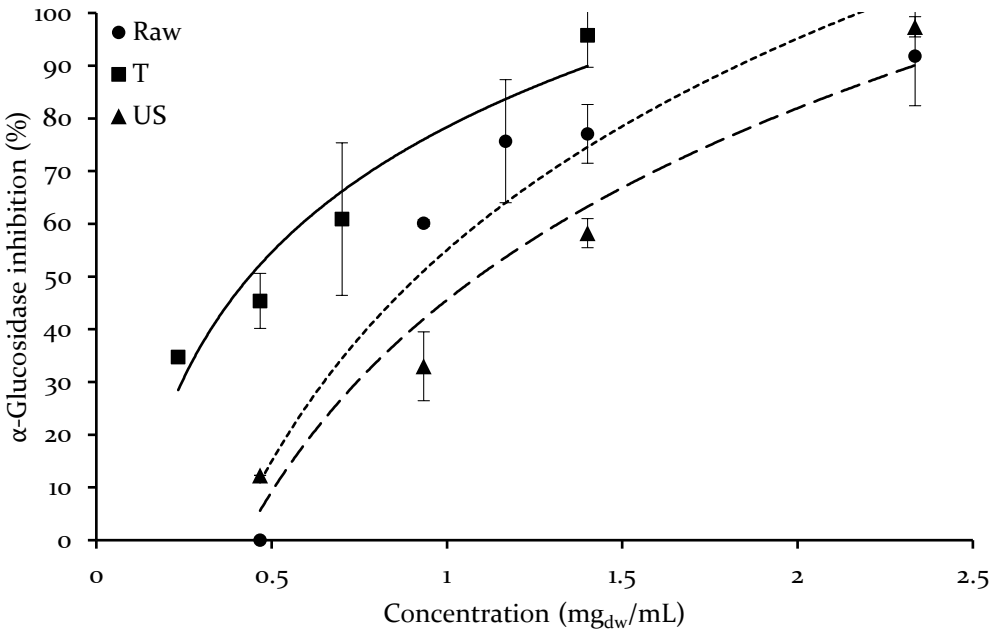


Figure 26. α-Glucosidase inhibitory activity of digested apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization, as a function of total solid concentration. Data fitting: lines, estimates; symbols, experimental data.

All digested samples inhibited α-glucosidase in a concentration-dependent manner but to a different extent. To rate the efficacy of digested sample in inhibiting the enzyme, the IC_{50}, i.e. the half-maximal inhibitor concentration, was computed and compared to that of acarbose, which is one of the most commonly used drugs for the treatment of type 2 diabetes (Table 25).

Table 25. IC_{50} (half-maximal inhibitory concentration) and acarbose equivalents of digested apple juice not pasteurized (Raw) or subjected to thermal (T) or ultrasound (US) pasteurization.

Sample	IC_{50} (mg/mL)	Acarbose equivalents ($IC_{50\ acarbose}/IC_{50\ inhibitor}$)
Raw	0.92	0.10
T	0.44	0.21
US	1.09	0.08
Acarbose	0.09	1

Thermal pasteurized apple juice was more effective than raw apple juice in inhibiting α-glucosidase upon *in vitro* digestion, while ultrasound pasteurization reduced the inhibitory capacity of apple juice (Figure 26 and Table 25). The ability of apple juice to inhibit α-glucosidase was previously assessed on undigested apple juice and was 6- to 40-fold lower

as compared to the digested samples (Table 22), indicating that the digestion process plays a key role in determining food functionality. The most effective sample, i.e. thermal pasteurized apple juice, also contained the highest concentration of total phenolic compounds in the digested sample (Figure 23). However, no correlation ($p > 0.05$) was found between the phenolic concentration and the IC_{50}. Even if several authors (Boyer & Liu, 2004; Hyson, 2011) attributed the antidiabetic effect of apple juice to the ability of phenolic compounds to inhibit α-glucosidase, the absence of a direct correlation between the phenolic content and the α-glucosidase inhibitory capacity was already pointed out in the undigested apple juice (Table 21 and Figure 19). This might suggest that not only phenolic compounds but also other molecules, such as those formed upon thermal treatment, may be involved in the antidiabetic effect of apple juice.

4.2.3.5 Serum albumin binding ability

As already pointed out, T and US pasteurized juices still accounted for a considerable concentration of phenolic compounds even upon digestion, although their profile was different. In particular, chlorogenic acid survived gastrointestinal conditions only in the US pasteurized apple juice, whereas it was completely depleted in the thermally pasteurized sample (Figure 23). To understand whether chlorogenic acid could be effectively carried to other target sites to elicit its many other health-related benefits, among which are hepatoprotective, cardioprotective, neuroprotective, anti-inflammatory, antimicrobial, and anti-hypertension effects (Naveed *et al.*, 2018), US pasteurized juice was analysed to assess the interactions occurring with a major transport protein in blood. As a model system human serum albumin (HSA) was used. This binding process was observed making use of the sensitivity of time-resolved fluorescence. The measurement of the fluorescence lifetime by its nature gives an absolute measure, which is concentration independent and thus not affected by photobleaching, sample dilution or changes in excitation intensity. Hence, this technique is highly suitable to monitor the binding of fluorescent components in the apple juice extract to HSA. A methodology similar to that previously applied to the binding of butterfly pea extract to BSA was employed (Hungerford *et al.*, 2019). In order to excite the extract a longer wavelength (378 nm) was selected, as using a shorter wavelength light (e.g. <295 nm) would also excite fluorescent amino acids in the HSA (notably tyrosine and tryptophan) as well as the extract (Figure 24). Therefore, when considering the spectral regions indicated in Figure 24, it is be region "A", which is principally associated with chlorogenic acid, that was monitored. The emission around 450 nm with excitation at 378

nm was explored using decay associated spectra, both before and after the kinetic study. This type of measurement has previously been used to investigate blue-green fluorescence in artichoke leaves and was linked with chlorogenic acid (F. Morales, Cartelat, Álvarez-Fernández, Moya, & Cerovic, 2005). The outcome of these measurements is shown in Figure 27.

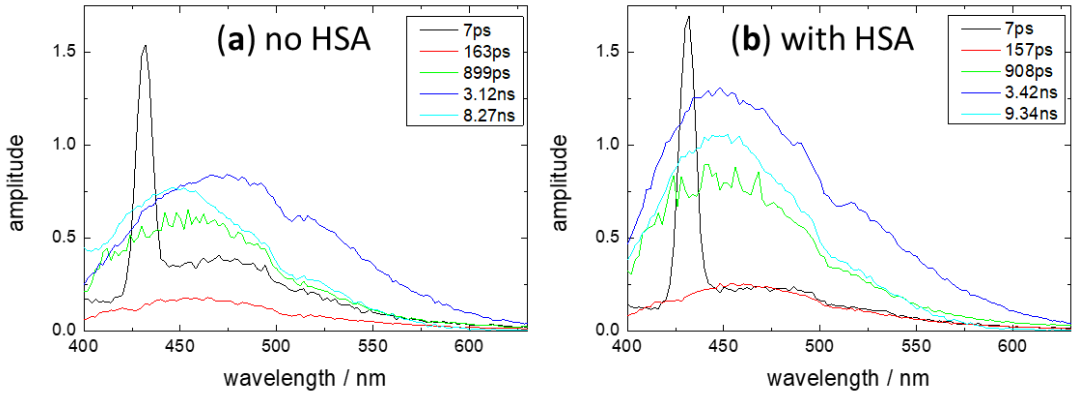


Figure 27. Decay associated spectra for US treated digested extract, both before (a) and after (b) the addition of HSA. The fluorescence lifetime associated with the spectra are shown. That of 7ps (half a time bin) was fixed in the decay analysis to account for scattered light and its associated spectra is dominated by a peak that can be attributed to Raman scattering from the solvent.

The results shown in Figure 27 indicate that the emission seen in the EEMs (Figure 24) is more complex and consists of several fluorescing species, with (at least) the presence of four principal emissions in addition to a short-lived one dominated by a Raman scattering feature (at 431 nm). This short-lived spectrum also appears to have an emission around 465 nm. Considering the extract prior to addition of HSA (Figure 27a) then an emission peaking ~458 nm is seen associated with a lifetime of 163 ps, this is a similar wavelength to the spectrum associated with a decay time of 899 ps. The spectra associated with longer lifetimes, 3.12 ns and 8.27 ns exhibit peak at ~468 nm and 450 nm respectively. These results are indicative of the presence of more than one fluorescing component.

After addition of HSA (Figure 27b) some notable changes in the decay associated spectra were observed. Overall, an increase in the emission intensity (close to 1.3 times) occurred and the lifetimes of the two longer-lived associated spectra increased. There were also significant changes in the peak emission wavelengths of the spectra associated with the 908 ps and 3.42 ns lifetimes (to 449 nm and 455 nm respectively). A hypsochromic shift is usually indicative of emission from a less polar environment (Lakowicz, 2006) and in this case can relate to fluorescent compounds in the extract locating to the interior of the HSA and is corroborated by the increase in the fluorescence lifetime. These observations are in keeping

with binding studies using fluorescent probes (Sudlow, Birkett, & Wade, 1975) and other work has indicated that the dominant binding site for both CGA and flavonoids is Sudlow's site I (Bolli *et al.*, 2010; Y. J. Hu, Chen, Zhou, Bai, & Ou-Yang, 2012).

This interaction process between the extract and HSA can also be followed making use of a "photo streaming" measurement. To this purpose, HSA was added to the US treated digested sample and two wavelengths (415 nm and 510 nm) were selected either side of the main emission peak to elucidate differences in behaviour of the extract since it is known to be a mixture of different compounds. Both intensity and lifetime data were obtained (Figure 28) and a region 1.5 seconds in duration around the addition point was investigated. In order to obtain sufficient photons to analyze (Köllner & Wolfrum, 1992) a resolution of 5 ms per point was used and the time-resolved decays were simply fitted to a single exponential decay model to elucidate differences.

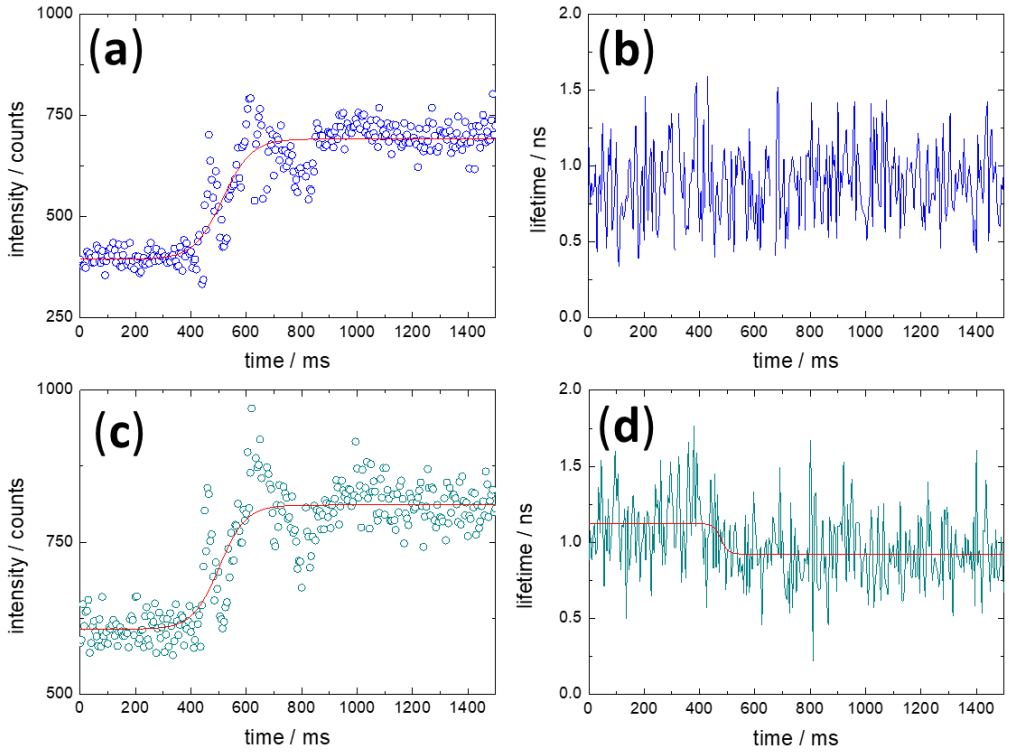


Figure 28. Time-resolved fluorescence data for the addition of HSA to US treated digested extract excited at 378 nm. The emission was monitored simultaneously at 415 nm (a & b) and 510 nm (c & d) with intensity data (a & c) and the lifetime obtained from a single exponential fitting model (b & d) shown.

Overall the results show an increase in intensity upon addition of the HSA (~1.75x at 415 nm and ~ 1.34x at 510 nm), which is in keeping with that observed in the decay associated spectra. At 410 nm no significant change in lifetime was seen, while a slight decrease was

observed at 510 nm. This can be because of the hypsochromic shift noted in the decay associated spectra in some of the longer-lived (908ps, 3.42 ns) associated spectra. The rate at which the fluorescence intensity (counts) changes is slightly higher at 415 nm (52.2 ms^{-1}) compared to that at 510 nm (48.1 ms^{-1}). It should be noted that other work has shown that the binding constant to HSA of the probable main fluorescing species (CGA and quercetin) show that CGA has a higher binding constant than quercetin (Y. J. Hu *et al.*, 2012; Knjazeva & Kaljurand, 2010). However, since the extract is complex in terms of having several fluorescing species it is difficult to make concrete comments in relation to the binding kinetics. It should also be kept in mind that as well as the species elucidated by their fluorescence in the wavelength range studied there are also compounds in the extract that can be binding, thus in fact competing with those fluorescent species, but because of their absorption and emission wavelengths go unnoticed in the study.

4.2.3.6 Energy density and electrical energy consumption

To estimate the efficiency of thermal and US pasteurization treatments the power and energy density for both the heating and cooling (to a final temperature of about 50 °C) phases were computed (Table 26). It appears that the US treatment required a much higher energy density despite its shorter treatment time. On the contrary, the energy density needed to cool down the US treated juice was lower, due to the lower temperature reached by the sample at the end of the heating phase. Table 26 also shows the electrical energy use of both treatments during the heating phase. US pasteurization resulted the most energy consuming treatment. Although its duration was lower, it must be born in mind that temperature control is needed during the whole US treatment, to mitigate the temperature increase that would occur.

Table 26. Energy density and electrical energy use during heating and cooling phases of thermal (T) and ultrasound (US) pasteurization of apple juice.

	Heating phase				Cooling phase			
	Power density (kW/m^3)	Energy density (MJ/m^3)	Duration (s)	Electrical energy use (MJ/m^3)	Power density (kW/m^3)	Energy density (MJ/m^3)	Duration (s)	Final temperature (°C)
Thermal pasteurization	max: 1588 average: 288	324	1120	520	max: -1084 average: -580	-174	310	51.6
US pasteurization	1160	696	600	4260	max: -607 average: -437	-55.1	120	51.3

4.2.4 Conclusions

The results obtained highlight that processing and digestion oppositely affected the phenolic content in apple juice: even if processing induced an increase in their concentration, they suffered a prominent decrease upon digestion. This was most pronounced in the US pasteurized sample. Still, after digestion both T and US pasteurized juices presented a higher concentration of phenolic compounds potentially available for intestinal absorption, as compared to the digested raw sample. Thus, since bioaccessibility was lower in pasteurized samples as compared to the raw one, it can be inferred that bioaccessibility alone cannot explain the joint effect of processing and digestion on compounds actually available for gut absorption. Moreover, even when phenolic compounds are effectively delivered at gastrointestinal level to elicit their bioactivity, their concentration is not correlated with the antidiabetic potential of apple juice, as observed in the case of the undigested juice.

Nevertheless, acquired results showed that the components available for intestinal absorption actively bind to the main blood transport protein (HSA) and can thus be delivered to other organs and tissues to carry out their bioactivity.

Based on the results obtained and considering that US pasteurization was the most energy wasting treatment despite its shorter duration, no univocal indication on the best pasteurization process to be applied to boost apple juice functionality can be surmised. Evaluating the effect of a technological intervention on food functionality necessarily requires considering all available data to avoid result misinterpretation. When carrying out studies relevant to food enrichment with bioactive compounds it is crucial to bear in mind that increasing their bioaccessibility does not necessarily mean increasing the concentration of compounds available for intestinal absorption, nor their functionality. This includes transport efficiency and bioactivity. Defining the desired concentration at the intestinal level, as well as in the target organs and tissues, would be helpful in developing the proper technological intervention to drive functionality by a customized approach, without neglecting the energy consumption point of view.

Chapter 5 Apple pomace

Apple pomace is the main by-product resulting from apple juice or cider production and consists of flesh, peels, seeds, and stalks. It accounts for 25–35% of the dry mass of apple and thus each year 10 million tons of apple pomace are obtained from apple juice production worldwide (Gullón, Falqué, Alonso, & Parajó, 2007). It is generally managed through the typical framework followed for other vegetable discards. Most commonly, it is disposed of or used to produce energy and bioethanol (Chatanta *et al.*, 2007). However, apple pomace still contains a great amount of high value-added compounds, such as dietary fiber (Gouw, Jung, & Zhao, 2017; Wolfe, Wu, & Liu, 2003) and polyphenols (Rodríguez, Jiménez, Fernández-Bolaños, Guillén, & Heredia, 2006).

These compounds are considered responsible for the health-promoting properties of apples (Boyer & Liu, 2004; Hanhineva *et al.*, 2010; Shoji *et al.*, 2017). Thus, in the last years the interest towards the recovery of such value-added compounds has considerably increased. Biochemical processing and chemical extraction have been proposed as alternative management strategies for apple pomace (Perussello, Zhang, Marzocchella, & Tiwari, 2017). Still, these are costly and require an efficient managing framework.

A promising approach to overcome these drawbacks is to reuse apple pomace, upon only negligible changes, as functional ingredients (Carson, Collins, & Penfield, 1994; Gouw *et al.*, 2017). Increasing research is currently focusing on this topic, due to the concomitant need to booster food functionality and increase the sustainability of the food system (Haghighi & Rezaei, 2013; Issar, Sharma, & Gupta, 2017; Mir, Bosco, Shah, Santhalakshmy, & Mir, 2017; Sudha, Baskaran, & Leelavathi, 2007).

However, in most of these studies the activity is limited to the characterization of reformulated food in terms of bioactive content. Knowledge is thus lacking about the bioavailability, intended as the actual efficacy in terms of functionality.

5.1 Use of apple pomace as a functional ingredient to reduce the glycemic response of short dough biscuits, and effect of their co-ingestion with different beverages

5.1.1 Aim of the study

The aim of the present study was to elucidate whether a partial replacement of wheat flour by apple pomace in a short dough biscuit, which represents a widely consumed bakery product, allows to decrease its glycemic response and thus to understand if this reformulation intervention could represent a dietary strategy for type 2 diabetes management. In addition, conventional and reformulated biscuits were administered to healthy rats in combination with apple juice or a soft drink simulating apple juice sugar content and the glycaemic response was evaluated. Part of the results here presented has been published:

- ✓ Alongi, M., Melchior, S., Anese, M. (2019). Reducing the glycemic index of short dough biscuits by using apple pomace as a functional ingredient. LWT - Food Science and Technology, 100, 300-305.

5.1.2 Material and methods

5.1.2.1 Apparatus and materials

The following apparatuses were used to carry out the experimental trials: vacuum oven (Bicasa, Milano, Italy), oven (Electrolux Professional, Pordenone, Italy), mill (Retsch, Hann, Germania), kneading machine (Kenwood, Milano, Italy), food laminating machine (Imperia & Monferrina, Roma, Italy), centrifuge (Brea, California, USA), spectrophotometer (Shimadzu Corporation, Kyoto, Japan), micro-centrifuge (Hittich, Tuttlingen, Germania), tristimulus colorimeter (Minolta, Osaka, Japan), texture analyzer (Instron LTD, High Wycombe, UK), freeze-drier (Edwards Alto Vuoto, Milano, Italia). α-Amylase from *Bacillus* sp., porcine pepsin, porcine pancreatin, porcine bile extract, amyloglucosidase from *A. niger*, L-(+)-arabinose, D-(-)-fructose, D-(+)-glucose, sucrose, HCl, NaOH, $CaCl_2(H_2O)_2$, Na_2CO_3, $NaHCO_3$, NaCl, KCl, KH_2PO_4, $MgCl_2(H_2O)_6$, $(NH_4)_2CO_3$ and total dietary fiber assay kit were purchased from Sigma Aldrich (Milano, Italy). Folin-Ciocâlteau's reagent, acetonitrile and

ethanol 98% (v/v) were purchased form Carlo Erba Reagents (Milano, Italy). Deionized water (System advantage A10®, Millipore S.A.S, Molsheim, France) was used.

5.1.2.2 Apple pomace preparation

Apple pomace (AP), consisting of peel, pulp, seeds, and stem, was recovered during the extraction of *Golden delicious* apple juice and immediately dehydrated by using a vacuum oven (75 °C and 0.1 MPa). Samples were milled (particle size < 200 μm) and stored at 20 °C and 5% RH under dark conditions until use.

5.1.2.3 Short dough biscuit preparation

Biscuits without AP (hereafter called "Control") or reformulated with 10% (hereafter called "R10") or 20% (hereafter called "R20") AP were prepared (Table 27).

Table 27. Composition of biscuit dough samples. Ingredients are listed according to the adding sequence.

Ingredients (% w/w)	Control	R10	R20
Egg	20.7	20.7	20.7
Sucrose	17.2	17.2	17.2
Sunflower oil	8.6	8.6	8.6
Wheat flour	51.6	46.4	41.3
AP	-	5.2	10.3
NaCl	0.2	0.2	0.2
Baking powder	1.7	1.7	1.7

In particular, wheat flour was replaced by 0 (control), 10 (R10) and 20 (R20) % (w/w) with AP on flour basis. The ingredients were mixed by a kneading machine and the dough was left to stand for 30 min at 4 °C. The dough was rolled out into a 2-mm layer and 50 mm diameter discs were obtained. Samples were baked at 140 °C for 15 min, removed from the oven, cooled to room temperature and sealed under vacuum in flexible polylaminate pouches for storage in dark conditions until analysis.

5.1.2.4 Sugars content

Sugars were quantified based on the method reported in paragraph 4.1.2.5.

5.1.2.5 Solids and dietary fiber

Total solids were determined by gravimetric method (AOAC, 1995). Soluble and insoluble dietary fiber was analyzed by using the total dietary fiber assay kit (AOAC, 1997), and expressed on dry weight basis.

5.1.2.6 Total phenolic content

Five g AP were extracted in 75 mL water, for 60 min at 100 °C. The mixture was cooled down and centrifuged at 7,000 *g* for 10 min at 4 °C to collect the apple pomace extract. Total phenol content was determined according to Singleton, Orthofer, and Lamuela-Raventos (1999). Briefly, 100 µL apple pomace extract was added to 900 µL water, 5 mL Folin-Ciocâlteau's reagent and 3.5 mL of Na_2CO_3 (150 g/L). The mixture was incubated for 2 h at 25 °C and absorbance was measured at 765 nm. Total phenolic content was calculated as $mg_{\text{gallic acid equivalent (GAE)}}/g_{\text{dry weight}}$.

5.1.2.7 Water-holding capacity (WHC) and oil-adsorption capacity (OAC)

WHC was determined according to Sudha, Baskaran, and Leelavathi (2007), with slight modifications. Aliquots of 0.05 g AP were mixed with 1 mL water in a microcentrifuge tube, centrifuged at 13,000 *g* for 30 min, and decanted the excess water. The sample was weighed, and WHC was expressed as $g_{\text{water}}/g_{\text{dry weight}}$. OAC was similarly determined, by using sunflower oil instead of water. OAC was expressed as $g_{\text{oil}}/g_{\text{dry weight}}$.

5.1.2.8 Color

Color analysis was carried out as described in paragraph 3.1.2.4.

5.1.2.9 Firmness and thickness

Dough firmness was determined by penetrating 3 mm a 20-mm-thick dough layer at 100 mm/min with a 6.2 mm cylindrical probe attached to a 0.1 kN unit. Biscuit firmness was determined by compressing 10 g sample at 150 mm/min speed with a ten-blade Kramer shear cell attached to a 5.0 kN unit. Force-distance curves were recorded (Automated Materials Testing System, Version 5, Series IX, Instron Ltd.), and firmness was taken as the maximum force required to compress samples. The thickness of biscuits before and after baking was also measured with a caliper (Metrica, Milano, Italy).

5.1.2.10 *In vitro* digestion

In vitro digestion was carried out according to the protocol proposed by Minekus *et al.* (2014) and as described in detail in paragraph 2.1.2.9, except for some changes. In particular, a secondary intestinal digestion phase was conducted by adding 0.1 mL amyloglucosidase to the digestion mix, which was maintained under stirring at 37 °C for 2 h. A sample was collected after 20, 60, 90 and 120 min from the beginning of the secondary intestinal phase, and the digestion was stopped by adding ethanol 98% (1:4 v/v). Samples were centrifuged at 10,000 ×*g* for 5 min at 4 °C. The supernatant was collected and analyzed for sugar content. White bread (62.3% flour, 34.6% water, 1.2% salt, 1.0% bakery yeast, 0.9% sugar, w/w) was purchased by a local producer, *in vitro* digested and analyzed for sugar content and represented the reference sample for glycemic index computation.

5.1.2.11 AUC and glycemic index computation

Glucose release during the secondary intestinal digestion phase was plotted against time and the area under curve (AUC) was obtained (Matthews, Altman, Campbell, & Royston, 1990). The glycemic index, defined as the ratio between the area under the glucose curve of a test food during 120 minutes after the consumption and the area under the glucose curve of reference food (i.e. white bread), was computed (Jenkins *et al.*, 1981; Wolever *et al.*, 1991) as reported in Equation 15.

$$GI = \frac{AUC_s}{AUC_r} \times 100 \qquad \text{Equation 15}$$

where AUC_s and AUC_r represent respectively the area under the glucose curve relevant to the sample, i.e. control, R10 or R20 biscuits, and to the reference food, i.e. white bread (Brouns *et al.*, 2005).

5.1.2.12 *In vivo* experiment

An experiment involving rats was carried out as reported in paragraph 4.1.2.10.
The glycemic response was assessed after the intake of glucose, Control or R20 were administered to the rats in order to provide 1.58 g/kg_{bw} available carbohydrate (Belobrajdic *et al.*, 2016), after being fluidized with 1 mL water. Further trials were carried out considering

the combination of Control with 1 mL apple juice (P_{90}, paragraph 4.1.2.2) or with 1 mL of a soft drink mimicking the sugar composition of apple juice (Table 20).

5.1.2.13 Sensory analysis

Thirty panelists were involved in the sensory evaluation under laboratory conditions on a single day. Judges were not trained on sensory analysis of biscuits but were experts in the use of the selected sensory method. A 7-point hedonic scale (1 low intensity, 7 high intensity) was used and panelists were instructed to compare the sample (R10 and R20) to the control, which was attributed 4 points. Each panelist evaluated all the samples. Seven parameters representative of quality, i.e. texture, crispiness, sweetness, sourness, shortbread flavor, baked flavor and fruit flavor, were considered, based on the literature (Popov-Raljić, Mastilović, Laličić-Petronijević, Kevrešan, & Demin, 2013).

5.1.2.14 Consumer response

Conjoint analysis was used to evaluate consumer preference towards biscuits by decomposing total preference in partial preferences relevant to independent product attributes (Pelsmaeker, Schouteten, Lagast, Dewettinck, & Gellynck, 2017; Sillani, Miccoli, & Nassivera, 2017). Five attributes of short dough biscuits containing AP were selected as discrete experimental variables and named "package size", "sugar", "fiber", "diabetes" and "glycemic index". Different levels were associated to each experimental variable (Table 28).

Table 28. Experimental variables defining biscuit attributes and relevant levels used for the conjoint analysis.

Experimental variable	Level
Package size	"Single portions"; "400 g"
Sugar	Absent; "Low sugar content"; "Without sugars"
Fiber	Absent; "High fiber"; "Source of fiber"
Diabetes	Absent; "Suitable for diabetics"
Glycemic index	Absent; "Low glycemic index"

Experimental variables were combined according to an orthogonal experimental design, obtaining twenty product profiles, which represented the information available to consumer on a possible biscuit label. A non-probabilistic sample of 300 biscuit consumers, equally

distributed among men and women (age 18–73), was recruited at the University of Udine, Italy. The response of consumers towards a biscuit label reporting different information was assessed, by asking consumers to fill up a structured questionnaire, indicating, for each product profile, their preference on a 1–100 scale, without any prior information about biscuit reformulation.

Consumers were also asked to indicate their weight and height, and these data were used to compute the Quetelet's index (Khosla & Lowe, 1967), according to Equation 16.

$$BMI = \frac{weight\ (kg)}{height\ (m)^2}$$ Equation 16

5.1.2.15 Statistical analysis

Results are averages of at least three measurements carried out on two replicated samples and are reported as means ± standard deviation for *in vitro* assays and as means ± standard error for *in vivo* trials ($n \geq 6$). Analysis of variance (ANOVA) was performed with significance level set to $p < 0.05$; the Bartlett procedure was used to test the homogeneity of variances, using R software, version 3.4.3 (The R Foundation for Statistical Computing, 2018).

For conjoint analysis, IBM SPSS Statistics 20 (Armonk, NY, USA) was used to calculate partial preference values and their relative importance, which were reported as means ± standard error, and the model goodness of fit (Pearson's R and Kendall's τ). t-Test was performed with $p < 0.05$.

5.1.3 Results and discussion

5.1.3.1 Physical and chemical properties of apple pomace

Results of chemical and physical analyses of AP are reported in Table 29.

Table 29. Dry matter, total (TDF), soluble (SDF) and insoluble (IDF) dietary fiber, fructose, glucose and sucrose, total phenolic content(TPC), color, water holding (WHC) and oil absorbing (OAC) capacity of apple pomace powder (AP).

Dry matter (%)		94.6 ± 0.1
Dietary fiber (%)	TDF	36.6 ± 0.2
	SDF	9.2 ± 0.2
	IDF	27.4 ± 0.1
Sugar ($mg/g_{dry\ weight}$)	Fructose	115.6 ± 0.5
	Glucose	44.4 ± 0.2
	Sucrose	14.9 ± 0.2
TPC ($mg_{GAE}/g_{dry\ weight}$)		1.1 ± 0.1
Color	L*	77.8 ± 0.5
	a*	2.6 ± 0.2
	b*	22.5 ± 0.4
Hydration properties	WHC ($g_{water}/g_{dry\ weight}$)	4.7 ± 0.2
	OAC ($g_{oil}/g_{dry\ weight}$)	1.0 ± 0.6

The sample presented a low moisture content, accounting for nearly 5%. Dry matter was mainly represented by TDF (37%), consisting of SDF and IDF with a ratio 1:3, in agreement with the literature (Carson *et al.*, 1994; Rana, Gupta, Rana, & Bhushan, 2015). TDF was in the same range as reported by other authors, i.e. 30 to 50% of total solids (Coelho & Wosiacki, 2010; O'Shea, Rößle, Arendt, & Gallagher, 2015; Sudha, Vetrimani, & Leelavathi, 2007; Yan & Kerr, 2013). The wide span of fiber concentration described in the literature can be attributed to the high matrix variability. Additionally, since apple pomace residues from apple juice processing, its composition is affected not only by the extrinsic (e.g. climatic conditions) and intrinsic (e.g. variety, ripeness degree) factors influencing apple growth, but also by storage conditions and dehydration techniques (Gullón *et al.*, 2007). The latter, and in particular thermal processing, are expected to induce phenolics degradation (Lu & Foo,

1997). TPC actually accounted for 1.06 $mg_{GAE}/g_{dry\ weight}$, in agreement with literature findings (Wolfe *et al.*, 2003). Consistently with literature data, fructose was the major component of AP sugars, accounting for 66 and 64% of total sugar content, respectively, while glucose and sucrose accounted for 25 and 24, and 9 and 12%, respectively (Gullón *et al.*, 2007). The golden yellow hue indicated by the color parameters was also observed by other authors (S. I. F. S. Martins, Jongen, & Van Boekel, 2001; Yan & Kerr, 2013).

The hydration properties of apple pomace fell in the same range reported by other authors, namely 1.6-8.4 $g_{water}/g_{dry\ weight}$ for WHC and 1.2-2.0 $g_{oil}/g_{dry\ weight}$ for OAC (Figuerola, Hurtado, Estévez, Chiffelle, & Asenjo, 2005; Rana *et al.*, 2015; Sudha, Baskaran, *et al.*, 2007). Apple pomace presented a 2.5-fold higher WHC than wheat flour (Joshi, Liu, & Sathe, 2015). The good hydration properties of apple pomace could be attributed to its higher content in soluble dietary fiber (more than 10%), when compared to wheat flour, in which SDF generally accounts for less than 5% (Taneyo, Di Silvestro, Dinelli, & Gianotti, 2017).

5.1.3.2 Effect of apple pomace use on biscuit physical, chemical and sensory properties

Apple pomace was used to partially substitute wheat flour in biscuits. The physical and chemical properties of biscuits prepared by replacing wheat flour with AP at 10 and 20% levels are presented in Table 30.

Table 30. Sugar content, thickness, firmness, and color of biscuits containing 0 (Control), 10 (R10) and 20 (R20) percent apple pomace powder (AP_{lab}) on flour basis. Means in the same column indicated by a common letter (a-c) are not significantly different ($p > 0.05$). n.d.: not detected.

Sample	Thickness (mm)	Firmness (kN)	Sugar (mg/g_{ss})			Color		
			Fructose	Glucose	Sucrose	L*	a*	b*
Control	4.4 ± 0.5^{a}	1.2 ± 0.1^{a}	n.d.	n.d.	46.3 ± 1.3^{b}	86.1 ± 0.5^{a}	5.0 ± 0.4^{b}	16.2 ± 0.8^{b}
R10	2.8 ± 0.2^{b}	0.8 ± 0.0^{c}	8.9 ± 0.1^{b}	n.d.	51.5 ± 1.4^{ab}	73.3 ± 0.4^{b}	10.8 ± 0.3^{a}	22.1 ± 0.6^{a}
R20	2.1 ± 0.1^{c}	1.0 ± 0.1^{b}	11.5 ± 0.0^{a}	n.d.	52.3 ± 0.1^{a}	72.4 ± 0.3^{c}	11.3 ± 0.1^{a}	21.6 ± 0.4^{a}

As the concentration of AP increased, the thickness of biscuits decreased, when compared to the conventionally formulated biscuit (i.e. control), in agreement with the literature (Sudha, Baskaran, *et al.*, 2007). The limited volumetric increase can be attributed to the strong WHC of apple fiber (Table 29), which in turn might be related to changes in dough firmness (Chen, Rubenthaler, Leung, & Baranowski, 1988). The latter significantly increased

when wheat flour was partially replaced by apple pomace, corresponding to 1.1 ± 0.1, 1.6 ± 0.1 and 1.7 ± 0.1 N for control, R10 and R20 respectively, potentially impinging volume increase during baking. In fact, firmness of R10 and R20 biscuits (Table 30) resulted lower ($p < 0.05$) than that of the control sample. Similarly, Matejová, Fikselová, Čurlej, and Czako (2016) observed a reduction in biscuit firmness when wheat flour was replaced with apple, buckwheat and grape pomaces. Such a difference is not expected to rely on moisture, which was comparable for all biscuits (i.e. 3.5 ± 0.6%) but could depend on other interactions occurring within the matrix. To this regard, Plazzotta, Sillani, & Manzocco (2018) reported that the water absorption capacity of fruit and vegetable fiber plays a role in determining the firmness of bakery products. In addition, the partial removal of wheat flour reduced gluten content, thus limiting gluten development during mixing and resulting in softer biscuits (Devisetti, Ravi, & Bhattacharya, 2015).

As expected, the control sample only contained sucrose, while the concentration of fructose, which was the major AP sugar (Table 29), increased as apple pomace content increased. Even if glucose was found in AP, it was not detected in reformulated biscuits, due to its depletion upon Maillard reaction occurring during baking (S. I. F. S. Martins *et al.*, 2001).

The lightness of AP-containing biscuits significantly decreased when compared to the control, while a* and b* concomitantly increased, indicating a more pronounced browning of biscuits containing apple pomace. This could be due not only to the use of an ingredient that originally has a different color from that of wheat flour, but also to the presence of glucose and fructose, which represent reactants of the Maillard reaction. The latter is well known to induce browning in baked foods, caused by the development of melanoidins and other Maillard reaction products (S. I. F. S. Martins *et al.*, 2001).

5.1.3.3 Effect of apple pomace use on glycemic response

Conventional and AP-containing biscuits were *in vitro* digested to assess the effect of reformulation on the predicted glycemic index. Figure 29 shows glucose concentration during the second intestinal phase of *in vitro* digestion.

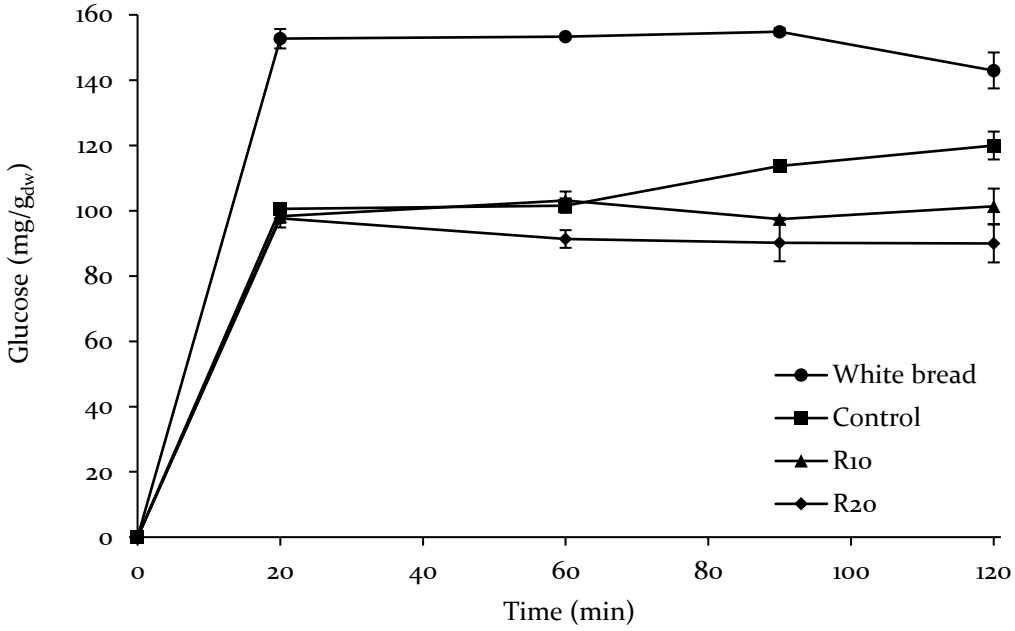


Figure 29. Glucose concentration as a function of time during the second intestinal phase, relevant to biscuits containing 0 (control), 10 (R10) and 20 (R20) percent of apple pomace (AP) on flour basis.

Glucose concentration increased during *in vitro* digestion for all samples, presenting a sharper growth during the first 20 min of the second intestinal phase. As expected, the maximum glucose concentration, accounting for 155 mg/g$_{dry\ weight}$ after 90 min, was recorded in white bread. Glucose concentration in control biscuits increased up to 120 mg/g$_{dry\ weight}$ after 120 min. On the contrary, the maximum glucose concentration recorded for AP-containing biscuits corresponded to 98 and 97 mg/g$_{dry\ weight}$ for R10 and R20, respectively, after 20 min.

Glucose concentration data collected during the second intestinal phase of *in vitro* digestion were used to estimate the glycemic index of control, R10, and R20 biscuits. The conventional biscuit (control) presented a glycemic index of 70.4 ± 0.2 and was thus classified as high glycemic index food. Substituting flour by 10 (R10) and 20 (R20) percent significantly ($p < 0.05$) reduced biscuit glycemic index to 65.7 ± 1.8 and 60.8 ± 1.9 respectively, thus ranking the product within the intermediate glycemic index foods (American Diabetes Association, 2004).

The reduction in the glycemic index, which resulted significant in biscuits with the highest AP content, can be attributed to the considerable TDF content of this by-product (Table 27). Total dietary fiber is well known to contribute to glycemic index reduction by several mechanisms. Soluble dietary fiber can increase matrix viscosity at gastrointestinal level,

contributing to the formation of a gel. The latter can envelop starch grains, protecting them from the amylolytic activity of digestive enzymes and thus impinging the release of free glucose, resulting in a reduced glycemic response (Brennan, 2005; Juvonen *et al.*, 2009). Despite insoluble dietary fiber does not directly influence postprandial glucose excursions, it plays a part in affecting the glycemic response, as it was demonstrated to affect gut transit time and was associated with a significant reduction of type 2 diabetes risk (Weickert & Pfeiffer, 2018; Wilfart *et al.*, 2007). Since AP presented a higher WHC than that reported in the literature for wheat flour, this by-product could entrap the water contained in the dough. Consequently, during biscuit baking, starch gelatinization would be partially prevented, inducing the retention of a high concentration of native starch. The latter can also be defined as resistant starch, since it is inaccessible to digestive enzymes (Miao, Jiang, Cui, Zhang, & Jin, 2015). It is noteworthy that resistant starch may consist of retrograded starch, physically inaccessible starch, starch-nutrient complexes, chemically modified starch (H. N. Englyst & Macfarlane, 1986). As a consequence, resistant starch cannot be hydrolyzed in the gastrointestinal tract to release free glucose, thus not contributing to the glycemic response (K. N. Englyst *et al.*, 1999).

Despite sugar content increased from 46 (control) to 60 (R10) and 65% (R20), a significant reduction in the glycemic index estimate was found when apple pomace was used to partially replace wheat flour. A further reduction in the glycemic index could thus be pursued by balancing the amount of sucrose used in biscuit formulation with sugars deriving from apple pomace.

To further investigate the antidiabetic potential of apple pomace, the glycemic response of short dough biscuits was also tested *in vivo* (Figure 30).

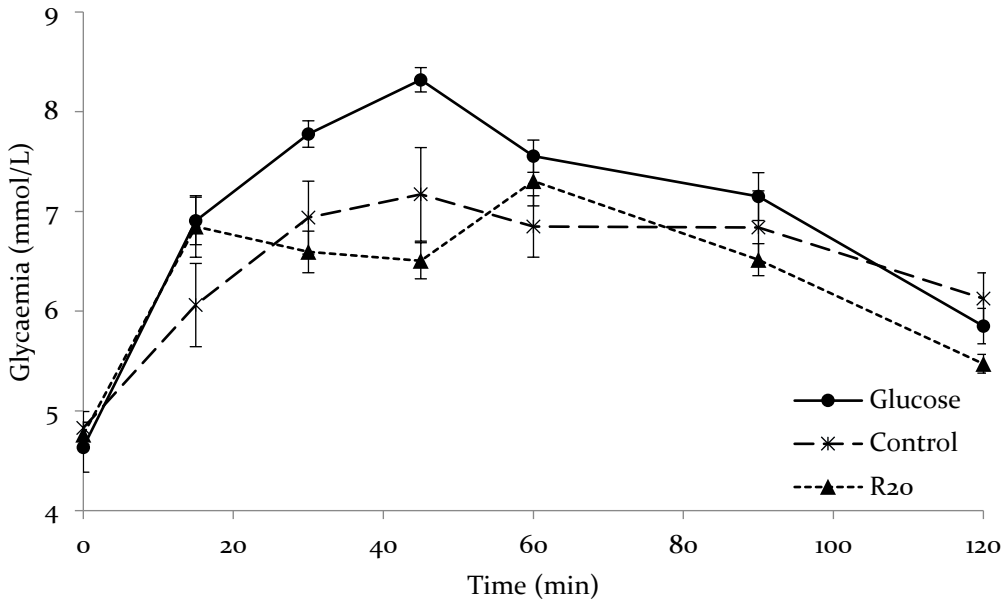


Figure 30. Glycaemic response of biscuits reformulated by replacing by 20% wheat flour with apple pomace (R20) and the conventional ones (Control), as compared to glucose.

The reformulation was confirmed to positively affect the glycemic response, as the glycemic peak was shifted at 60 min and the AUC slightly decreased, accounting for 13,997 ± 138, as compared to the Control one (14,232 ± 429).

Due to the promising results acquired in terms of antidiabetic properties of both thermal pasteurized (P_{90}) apple juice (Figure 26) and apple pomace (Figure 29), further research was carried out. In particular, to understand whether the glycemic response of biscuits could be modulated not only by reformulation with apple pomace but also through the concomitant consumption of apple juice, due to its antidiabetic potential, biscuits were administered together with apple juice or with a soft drink mimicking apple juice sugar composition (Figure 31).

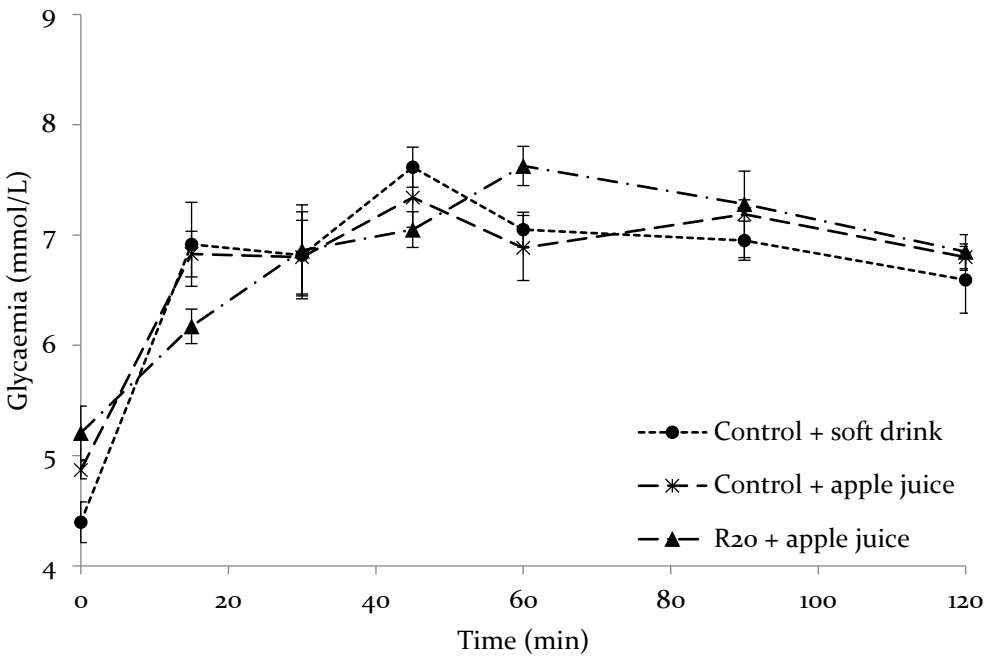


Figure 31. Glycemic response of the conventional biscuit consumed combined with a soft drink or with apple juice.

As shown in Figure 31, different glycemic responses were observed upon the co-ingestion of the conventional biscuit with the soft drink or with apple juice. The former induced a higher peak as compared to the latter, which in turn produced a lower and flatter glycemic curve. However, the glycemic peak was observed after 45 min in both cases, as previously observed also for the Control biscuit consumed alone (Figure 30). On the contrary, when R20 biscuit was ingested with apple juice, the shift in the glycemic peak observed upon the reformulation intervention (Figure 30) was maintained and resulted added up to the flattening in the glycemic curve (Figure 31).

Based on these results, it can be inferred that properly combining a product reformulated to lower its glycemic response (Figure 30) with a beverage known for its ability to inhibit α-glucosidase (Figure 26) could result in a meal with a bolstered antidiabetic potential. Still, these are preliminary results and further research is required to elucidate the effect of reformulated biscuits and apple juice combination towards the glycemic response.

5.1.3.4 Consumer acceptability and communication effect

Besides affecting the functional properties of short dough biscuits (Figure 29, Figure 30 and Figure 31), the reformulation intervention also induced changes in the physical and chemical properties of biscuits (Table 30). To understand if and to what extent this affected the

perception of biscuit sensory properties, some representative descriptors (Popov-Raljić *et al.*, 2013) were evaluated based on a hedonic scale and results are reported in Figure 32.

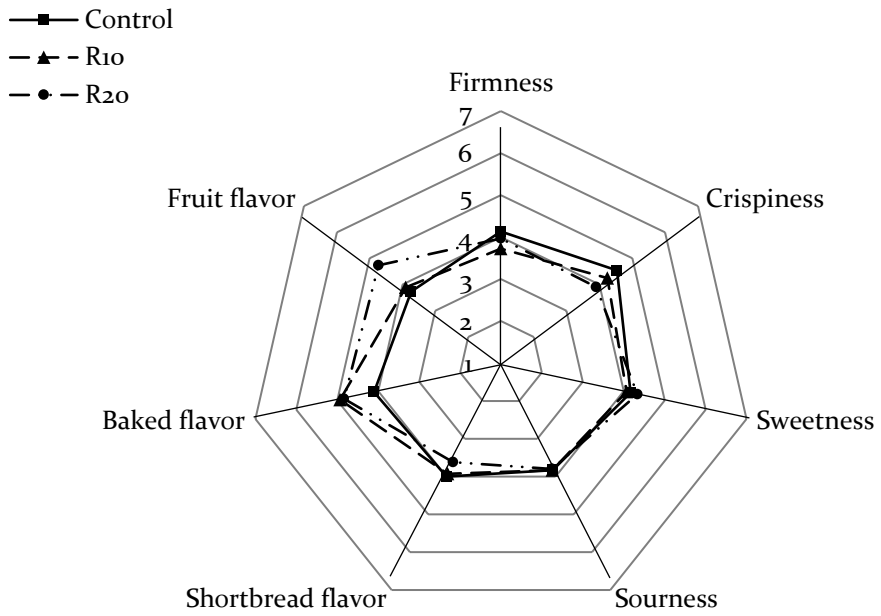


Figure 32. Sensory scores attributed to biscuits containing 0 (control), 10 (R10) and 20 (R20) percent apple pomace (AP) on flour basis.

Wheat flour replacement by AP did not affect the perception of most descriptors, such as firmness, crispiness, sweetness, sourness, and shortbread flavor (Figure 32). On the contrary, the baked and fruit flavors were differently perceived in biscuits containing 10 and 20% APP. Both reformulated biscuits presented significantly higher scores ($p < 0.05$) for the baked flavor when compared to the control, probably due to the faster evolution of the Maillard reaction in AP-containing biscuits, leading to characteristic flavors (S. I. F. S. Martins *et al.*, 2001).

The fruit flavor resulted significantly more intense ($p < 0.05$) in biscuits containing the highest amount of AP (20%), while the lower concentration (10%) did not affect its perception when compared to control, indicating that the addition of a by-product originated from fruit processing was not perceivable at low concentration. To this regard, Dourado *et al.* (2014) did not observe significant changes in the sensory profile of biscuits in which wheat flour was replaced by 8 to 14% with apple pomace, while changes in the sensory profile of bakery goods were observed by other authors when a higher apple pomace concentration was used (de Toledo, Nunes, da Silva, Spoto, & Canniatti-Brazaca, 2017).

Although the promising results relevant to the ability of reducing the glycemic response thus positively affecting type 2 diabetes (Figure 29 and Figure 30), and the overall positive

sensory outcomes (Figure 32), a critical issue determining the effective delivery on the market of reformulated biscuit is represented by the labelled information, which indeed affects the reaction of consumers towards their consumption.

Conjoint analysis was thus applied to assess consumers' response towards a biscuit label reporting different information. Claims associated with the content of compounds potentially affecting type 2 diabetes, as well as claims more directly referring to the disease, were considered (Table 28). Partial preference coefficients and relative importance of each label information in defining consumer response are reported in Table 31.

Table 31. Partial preference coefficients and relative importance of different label information in defining consumer preference. Pearson's R = 0.98 ($p < 0.0001$); Kendall's $\tau = 0.85$ ($p < 0.0001$); Kendall's τ for controls = 1.00 ($p < 0.05$).

Information	Partial preference coefficient	Relative importance
400 g	1.16 ± 0.33	15.03 ± 0.86
Single portions	-1.16 ± 0,33	
Suitable for diabetics	-2,38 ± 0.33	17.41 ± 0.71
Absent	2,38 ± 0.33	
Low glycemic index	0.11 ± 0.33	14.81 ± 0.63
Absent	-0.11 ± 0.33	
Low sugar	4.03 ± 0.44	27.58 ± 0.78
Sugar free	-0.98 ± 0.51	
Absent	-3.05 ± 0.51	
Source of fiber	1.13 ± 0.44	25.18 ± 0.80
High fiber	2.75 ± 0.51	
Absent	-3.88 ± 0.51	

The partial preference coefficient presented positive or negative values, the former indicating a higher preference as compared to the latter.

Information about the package size was provided, since controlling the portions represents a key feature to manage diet related diseases, such as diabetes (Pedersen, Kang, & Kline, 2007). Surveyed consumers preferred the 400 g instead of single portions. It should be kept in mind that the consumers involved in the survey were not overt diabetics. Still, nearly 20% of the interviewed consumers resulted overweight or even obese, according to their BMI

class (WHO, 2019). As obesity represents one of the major risk factors for type 2 diabetes, further research is required involving overt diabetics under treatment, to understand how consumer response changes between diseased subjects and those healthy or unaware of their disease.

Interestingly, consumers preferred not to be provided with information directly referring to diabetes ("suitable for diabetics"), whereas the information relevant to the glycemic index did not affect consumer response in a significant manner.

Considering sugar content, which indirectly refers to diabetes as its intake negatively affects diabetes, consumers preferred the "low sugar" to the "sugar free" claim, indicating a sugar content below 5%, and 0.5%, respectively (Reg. (EU) n. 1924/2006). The lower preference evidenced in the case of the absence of sugar, could lie in the less natural perception of the product by consumers (Siegrist & Sütterlin, 2017). In fact, short dough biscuits without sugars generally present sweeteners in their formulation, which might be negatively perceived by consumers. These tend to look for a compromise between health and naturalness perception, as well as taste, that is the crucial feature affecting food choices (Giordano *et al.*, 2018; Kaur & Das, 2011).

On the contrary, considering the fiber, which is known to positively affect glycemic response and was contained in high concentration in apple pomace, consumers preferred the "high fiber" over the "source of fiber" claim referring to a concentration ≥ 6% and ≥ 3%, respectively (Reg. (EU) n. 1924/2006).

Interestingly, it can be noticed that on the one hand consumers preferred not to be provided with information directly referring to the disease, but on the other hand they expressed a higher preference when information about nutritional features affecting the disease were provided. To this regard, it should be kept in mind that consumers generally prefer information highlighting the health promoting effect in a general manner in spite of directly relating to a specific disease (Schnettler *et al.*, 2019). In fact, consumers generally do not dispose of the knowledge required to understand specific and technical information and to relate it to a beneficial effect towards their own health (Bech-Larsen & Scholderer, 2007).

Table 31 also shows the relative importance of each attribute, indicating the importance given by consumers during the expression of their preference. Information relevant to sugar content resulted to most influencing, followed by fiber content. The absence of indication about the disease played a crucial role in determining consumer preference as well. On the contrary, lower importance was attributed to the glycemic index, as well as to the package size.

In addition, partial preference and relative importance of each information were compared among BMI classes. No differences were observed, indicating that the information provided equally affected all the population, which in general preferred information about the nutritional features instead of the disease, in agreement with literature findings (Bimbo *et al.*, 2017; Van Kleef *et al.*, 2005). This means that marketing campaigns promoting functional foods by highlighting the content of bioactive compounds and the overall effect on wellbeing can play a role not only in the management of diet-related diseases but also towards the maintenance of a healthy status.

Overall, these results pointed out that the choice of information provided to consumers plays a critical part in determining the success of functional foods on the market. Choosing a nutritional claim must thus consider not only the target population, such as diabetics and obese subjects in this case, but also healthy persons consuming functional foods to maintain their wellbeing.

5.1.4 Conclusions

Results acquired in the present study demonstrated that a partial replacement of wheat flour with apple pomace significantly reduced the glycemic index of biscuits. Reusing vegetable by-products rich in dietary fiber could thus not only contribute to reduce food discards, thus limiting the environmental impact of food production, but could also represent a valuable approach to develop dietary strategies for the management of type 2 diabetes. To this regard, further research is needed to define the composition of processing by-products in terms of bioactive compounds, as well as to minimize their degradation upon the processing interventions required to convert by-products into ingredients.

In addition, acquired results showed that the combination with other foods or drinks crucially affects the glycemic response, suggesting that besides reformulation and processing interventions, attention should be paid to the dietary habits, as these play a key role in steering the efficacy of newly developed functional foods.

Chapter 6 General discussion

6.1 Introduction

Functional food development relies on two major technical aspects, namely the design and the evaluation. The former is based on technological interventions, whose impact on food quality must be assessed. Such activity is principally task of food technologists, which however often neglect the impact of physiological functions on food functionality. On the other hand, during the evaluation, the efficacy of functional foods is measured as affected by physiological functions. This activity is carried out by nutritionists and clinicians which often consider simplified model systems, thus neglecting the technological history of the food under investigation.

Thus, although several studies have already been published on functional foods, there is a gap between the design and the evaluation steps.

Matching the technological and the physiological viewpoint within this Ph.D. thesis produced new findings contributing to the knowledge required for the development of tailor-made functional foods.

This approach was applied to different vegetable food matrices, i.e. turmeric, coffee and apple derivatives, known for their health-promoting effects and in particular for the antidiabetic capacity. Design interventions included formulation, conventional and unconventional processing, as well as combinations with other foods or drugs. Functionality evaluation was carried out by both *in vitro* and *in vivo* trials. In particular, the phenolic compound bioaccessibility, the antidiabetic potential, intended as inhibitory capacity against α-glucosidase, acarbose interaction and impact on glycemic response, and the serum albumin binding were considered as bioactivity markers.

The pursued approach brought about some innovative aspects, potentially representing a starting point for future research directions in the field of functional foods.

6.2 Main findings

Chapter 2, confirmed that oil enrichment with turmeric and its structuring significantly affect curcuminoid bioaccessibility and lipolysis kinetics. Gel strength was the critical factor affecting the rate and extent of lipolysis whereas differences in the gelling agent modified curcuminoid bioaccessibility. A proper choice of oleogelation conditions could thus represent a promising strategy to reduce fat uptake while improving the bioaccessibility of loaded lipophilic bioactive molecules.

Chapter 3 highlighted the impact of different roasting intensity, as well as the combination with milk by HPH homogenization, on the phenolic compound bioaccessibility and the antidiabetic potential of coffee. Even though roasting produced a considerable decrease in the concentration of phenolic compounds, these were more resilient to digestion, which eventually nullified changes induced by roasting. In addition, the more intense was the roasting degree, the more pronounced was the antidiabetic potential of coffee, in terms of ability to inhibit α-glucosidase, suggesting that other compounds formed upon the thermal treatment, such as Maillard reaction derivatives, could play a role in affecting coffee antidiabetic ability. Properly combining milk fat and pressure positively affected both phenolic compound bioaccessibility and the potential of coffee to reduce type 2 diabetes risk.

Chapter 4 evidenced how different pasteurization interventions differently affected phenolic compound bioaccessibility and the antidiabetic potential of apple juice. Still, no straightforward matching was found between phenolic content and antidiabetic potential, fostering the hypothesis that other compounds formed upon thermal treatment may be responsible for the antidiabetic effect.

Chapter 5 demonstrated the efficacy of reusing the processing by-product deriving from apple juice production, i.e. apple pomace, as a functional ingredient to reduce the glycemic index of bakery foods, improving not only food functionality but also boosting the sustainability of this production.

Based on the results acquired during the research activity, the following can be considered as the major findings of this Ph.D. thesis:

- Both conventional (e.g. coffee roasting, apple juice pasteurization) and unconventional (e.g. turmeric-rich oil gelation, coffee and milk high-pressure homogenization) technological interventions may improve food functionality.

However, different and opposite effects may be obtained, depending on the intensity of the technological treatment as well as on the food matrix under investigation.

- Nor the antioxidant activity neither phenolic compounds can be considered the main responsible for food functionality. This may be delivered by other compounds, such as those formed upon thermal processing (e.g. melanoidins).
- The interactive behavior between food (i.e. apple juice) and drug (i.e. acarbose) changes as a function of the system concentration, resulting antagonistic in a packed system and synergistic in a dilute one.
- Reusing vegetable by-products (i.e. apple pomace) allows not only to improve the sustainability of food production but can also contribute to improving the functional properties of some foods, such as reducing the glycemic response of bakery goods.
- Even when an effective functional food is obtained, a critical point lies in the identification of the most suitable labeled information relevant to functionality, to drive consumer choices towards product consumption. Not only diseased subjects but also healthy consumers should be addressed, pursuing a prevention strategy.

6.3 Innovative aspects

The approach followed during the progress of this Ph.D. thesis presented some innovative aspects pointing out key features relevant to the study of functional foods. In particular, there is a need to:

- **Study the complex matrix instead of bioactive extract**. Studying the functional properties of the whole food leads to different results as compared to those available in the literature on simplified model systems that generally consider bioactive compound extracts. The whole matrix plays a crucial role due to interactions occurring among the different components.
- **Investigate food-food and food-drug interactions**. Besides interactions occurring among components within a single food matrix, attention should be focused also on those occurring between different foods. As these are generally consumed within a meal, studying the functionality as affected by the meal composition results essential to identify possible interactive behaviors. This consideration can be extended to the interactions between foods and drugs. Identifying synergistic windows could allow decreasing drug dosage thus reducing side effects connected with their intake. This approach could represent a starting

point to develop integrated strategies for managing and preventing food-related chronic diseases.

- **Consider functionality as affected by technological interventions and physiological functions**. Concomitantly considering the technological history of a food product and the changes induced by physiological mechanisms upon its ingestion represents the foundation on which strong and reliable knowledge about food functionality can be built.
- **Identify bioactivity indicators and mechanisms**. Indicators of food functionality should be considered in the light of overall available data to prevent misinterpretation. Bioaccessibility provides only partial information, as it does not provide any indication about the actual bioactivity, nor about the mechanisms underlying functionality.
- **Define a target**. Designing foods to deliver specific functionalities should not only focus on increasing the bioaccessibility of single bioactive compounds but should aim at maximizing bioactivity. Identifying the bioactive molecule and its mechanism, and defining the desired concentration at the intestinal level, as well as in the target organs and tissues, would be helpful in developing the proper technological intervention to drive functionality by a customized approach.

6.4 Future directions

Although *in vivo* trials represent the most accurate tool to assess functional outcomes, they are not able to provide insights into the mechanisms by which these outcomes are generated. In other words, they perform as a black box in which what is happening and how it leads to a certain result is not visible.

To understand the mechanism lying behind food functionality, *in vitro* tests are required. However, these should be carried out in a wider framework, by considering a modeling approach. As models allow not only to understand, but also to predict and control a phenomenon, this approach would help to optimize technological interventions on a theoretical basis, through *in silico* experiments, by identifying the most promising solutions to be tested *in vitro* and *in vivo*, thus saving resources.

A priori knowledge about the exact mechanism underlying a certain function at the physiological level and how it is affected by the interactions among food components, by

the technological interventions and by the digestion process is essential to steer food design and to ultimately obtain tailor-made functional foods.

6.5 Main conclusion

Food functionality was demonstrated to be dramatically affected by the food matrix, the technological interventions, intended as formulation and processing, as well as by co-ingested components and by the digestion process.

Thus, designing foods with healthy properties must take into account not only the knowledge of technological interventions, including formulation, processing, type of packaging and storage conditions, but also the expected consumption circumstances (e.g. co-ingestion with other foods or drugs) and the physiological processes occurring upon consumption.

Acquired results demonstrated the efficacy of some technologies in bolstering the antidiabetic properties of foods, as well as the potential to reduce drug dosage by proper combinations with functional foods, paving the way for new approaches to managing food-related chronic diseases.

Still, the molecules and the mechanisms underlying functional outcomes are partially unrevealed. This knowledge would provide a tool to steer functionality by targeted interventions, based on a holistic approach.

As understanding the mechanisms means learning from nature and engineer it to deliver specific functions, teamwork encompassing all the actors involved in the functional food development cycle is required, to successfully deliver on the market tailor-made functional foods.

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About the author

Marilisa Alongi, after attending the scientific high school, enrolled at the University of Udine in the Food Science and Technology course. During her bachelor's degree, she was granted an Erasmus scholarship and spent a semester at the University of Barcelona. She earned the master's degree in Food Science and Technology in 2015 with the highest marks, presenting a dissertation on the impact of technological interventions and shelf life extension on domestic food waste, supervised by Professor Lara Manzocco. She was awarded as "Best student", in 2015, and "Best graduated", in 2016, of the master's degree course in Food Science and Technology of the University of Udine. She was hired as a research fellow from 2015 to 2016 by the Food Technology Research Group of the University of Udine and started working on food functionality as affected by technological interventions. In 2016 she was awarded a research doctorate scholarship in Food and Human Health by the same University, under the supervision of Professor Monica Anese, presenting a project entitled "Healthy food development through novel sustainable processing and formulation". From October 2018 to February 2019 she was a visiting Ph.D. student at the Riddet Institute of Massey University, in New Zealand. During her Ph.D. she held seminars relevant to her research activity within the bachelor's and master's degree study courses in Food Science and Technology at the University of Udine. She assisted professors in examinations for invigilation and marking, and supervised bachelor and master students during their dissertation work. She also attended at several national and international conferences, presenting oral and poster contributions on her research activity. She was awarded with the "What for?" prize granted by Federalimentare within the "XXIV Workshop on the Development in the Italian Ph.D. Research on Food Science, Technology and Biotechnology", held in September 2019 in Florence, Italy, for the doctoral thesis with the most evident innovative and technological transfer impact and for the most effective communication of results.

Publications relevant to the Ph.D. activity

Publications on international peer reviewed journals

Calligaris, S., **Alongi**, M., Lucci P., Anese, M. (2020). Effect of oleogelator type on lipolysis kinetics and curcuminoid bioaccessibility upon *in vitro* digestion of sunflower oil-based oleogels. *Food Chemistry*. *DOI: 10.1016/j.foodchem.2019.126146.*

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In progress manuscripts

Alongi, M., & Anese, M. α-Glucosidase inhibitory capacity of *in vitro* digested coffee as affected by roasting. *Paper drafting.*

Alongi, M., Sillani, S., Verardo, G, Gorassini, A., Degrassi, C., & Anese, M. Effect of apple pomace containing biscuits and their co-ingestion with apple juice on the glycaemic response in healthy rats, and consumer acceptability. *Paper drafting.*

Additional publications

Alongi, M., Sillani, S., Lagazio, C., & Manzocco, L. (2019). Effect of expiry date communication on acceptability and waste of fresh-cut lettuce during storage at different temperatures. *Food Research International*, *116*, 1121-1125.

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Contributions to national and international conferences

Calligaris, S., **Alongi**, M., Lucci, P., Anese, M. (2019). Impact of gelator type and oleogel structure on lipolysis kinetics and curcuminoid bioaccessibility of sunflower oil-based oleogels. *17th Euro Fed Lipid Congress and Expo, Seville, Spain*. Oral communication.

Alongi, M., Anese, M. (2019). Effetto dei trattamenti di pastorizzazione sulle proprietà funzionali di succo di mela. *1st Workshop of the Multidisciplinary Group on Active Ageing of the University of Udine. Udine, Italy*. Oral communication*.

Alongi, M. (2019). Healthy food development through novel sustainable processing and formulation. *XXIV Workshop on the Development in the Italian Ph.D. Research on Food Science, Technology and Biotechnology, Florence, Italy*. Oral communication*.

Alongi, M., Melchior, S., Anese, M. (2019). Reducing the glycemic index of cookies by using apple pomace as a functional ingredient. *8th International Symposium on Delivery of Functionality in Complex Food Systems, Porto, Portugal*. Oral communication*.

Alongi, M., Calligaris S., Anese M. (2019). Coffee brew formulation and processing affect chlorogenic acid bioaccessibility and α-glucosidase activity. *8th International Symposium on Delivery of Functionality in Complex Food Systems, Porto, Portugal*. Poster.

Manzocco, L., **Alongi**, M., Maldonado-Simán, E. (2019). Acceptability and domestic waste of minced meat: a comparison between Italian and Mexican consumers. *9th Shelf Life International Meeting, Naples, Italy*. Poster.

Alongi, M. (2018). Effect of coffee and apple juice conventional processing on their inhibitory capacity against α-glucosidase. *XXIII Workshop on the Development in the Italian Ph.D. Research on Food Science, Technology and Biotechnology, Oristano, Italy*. Poster.

Alongi, M., Anese, M. (2018). Apple pomace as a source of bioactive compounds to reduce the glycemic index of cookies. *2nd Workshop on Food Waste Recovery & Open Innovation, Stuttgart, Germany*. Poster.

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Alongi, M., Anese, M. (2017). Investigating the *in vitro* inhibitory activity of roasted coffee against α-glucosidase. *Fourth International Congress on Cocoa, Coffee and Tea, Turin, Italy*. Poster.

Alongi, M. (2017). Healthy foods development through novel sustainable processing and formulation. *XXII Workshop on the Development in the Italian Ph.D. Research on Food Science, Technology and Biotechnology, Bolzano, Italy*. Poster.

Alongi, M., Manzocco, L., Lagazio, C., Sillani, S., Nicoli, M. C. (2017). Does food technology have a role in tackling food wasting? The case of fresh-cut salad. *International Conference on Food Innovation, Food Innova, Cesena, Italy*. Poster.

Alongi, M., Manzocco, L., Lagazio, C., Sillani, S., Anese, M., Nicoli, M. C. (2016). Development of a methodology to estimate domestic food waste: the case of fresh-cut salad. *4th International ISEKI Food Conference, Vienna, Austria*. Poster.

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Manzocco, L., **Alongi,** M., Anese, M., Sillani, S., Nicoli, M.C. (2016). Effect of temperature abuse on domestic food waste: the case of fresh-cut salad. *6th International Conference Cold Chain Management, Bonn, Germany*. Oral communication.

Manzocco, L., **Alongi**, M., Sillani, S., Lagazio, C. (2015). Effect of shelf life extension on consumer fresh-cut salad waste. *7th Shelf Life International Meeting, Monza, Italy*. Oral communication.

** Personally delivered.*

Awards

September 2019	"What for?" award granted by Federalimentare within the *XXIV Workshop on the Development in the Italian Ph.D. Research on Food Science, Technology and Biotechnology* (Florence, Italy) for the doctoral thesis with most evident innovative and technological transfer impact and for the most effective communication of results.
July 2016	"Best graduated" award of the master's degree course in Food Science and Technology of the University of Udine.
July 2015	"Best student" award of the master's degree course in Food Science and Technology of the University of Udine.