Università degli Studi del Piemonte Orientale

"Amedeo Avogadro"

Dipartimento di Scienze del Farmaco

PhD Course in Chemistry & Biology

XXX cycle

Formulation and characterization of spray-dried polyphenol-rich ingredients for functional foods

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Supervised by Prof. Marco Arlorio

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If at first you don't succeed,
try, try again!

W. E. Hickson

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1. Introduction

1.1 Functional foods

The focus of science applied to nutrition has changed over the centuries. In the past, food was considered primarily a source of energy and nutrients necessary for the daily functioning, and its intake was merely aimed at avoiding nutrient deficiencies. Successively, the taste, the flavour and the texture of food raised of importance, until they became the priority of the food sector operators, redirecting their research towards new targets. In the last years, feeding really became nutrition, and started to increasingly represent a way to be healthy, shifting the interest of food science and technology towards healthy foods (Ghosh, Das, Bagchi, & Smarta, 2012). Over 2500 years ago Hippocrates already said "*Let food be the medicine and medicine be the food*", opening to a new vision of food related to health. Even in Asia there is an old tradition, dating back to 1000 BC and long considered a popular belief, that attributes medicinal and health functions to certain foods and botanicals. Nevertheless, the first currently acknowledged healthy food was born in 1980 in Japan (El Sohaimy, 2012). The increasing interest and the rapid growth in the market of healthy foods led to their definition as "functional foods", even though a harmonized regulatory framework still lacks in many countries.

According to the International Food Information Council, *functional foods are foods capable of providing health benefit beyond basic nutrition* (IFIC Foundation, 1995). This concept is closely related to that of *health claim*, which in turn is different from the nutrition claim.

In the international guidelines of the *Codex Alimentarius* (CAC/GL 23-1997):

- *nutrition claim* is defined as "any representation which states, suggests or implies that a food has nutritional properties, including but not limited to the energy value and to the content (high or low) or absence of one or more substances". The only nutrition claims permitted on the label are those relating to energy, lipids, satured fats, cholesterol, sugars, sodium, protein, vitamins,

minerals and dietary fiber, for which exist Nutrient Reference Values (NRVs), such as 'low cholesterol', 'free from sodium', etc (**Tab. 1**).

Table 1. Table of condition for nutrient content claims (*Codex Alimentarius*)

COMPONENT	CLAIM	CONDITIONS (not more than)
Energy	Low	40 kcal per 100 g (solids) or 20 kcal per 100 mL (liquids)
	Free	4 kcal per 100 mL (liquids)
Fat	Low	3 g per 100 g (solids) 1.5 g per 100 mL (liquids)
	Free	0.5 g per 100 g (solids) or 100 mL (liquids)
Saturated fat	Low	1.5 g per 100 g (solids) 0.75 g per 100 mL (liquids) And 10% of energy from saturated fat
	Free	0.1 g per 100 g (solids) 0.1 g per 100 mL (liquids)
Cholesterol	Low	0.02 g per 100 g (solids) 0.01 g per 100 mL (liquids)
	Free	0.005 g per 100 g (solids) 0.005 g per 100 mL (liquids)
Sugars	Free	0.5 g per 100 g (solids) 0.5 g per 100 mL (liquids)
Sodium	Low	0.12 g per 100 g
	Very low	0.04 g per 100 g
	Free	0.005 g per 100 g
COMPONENT	**CLAIM**	**CONDITIONS (not less than)**
Proteins	Source	10% of NRV per 100 g (solids) 5% of NRV per 100 g (liquids) or 5% of NRV per 100 kcal or 10% of NRV per serving
	High	2 times the value for "source"
Vitamins and Minerals	Source	15% of NRV per 100 g (solids) 7.5% of NRV per 100 g (liquids) or 5% of NRV per 100 kcal or 15% of NRV per serving
	High	2 times the value for "source"
Dietary Fibre	Source	3 g per 100 g or 1.5 g per 100 Kcal or 10 % of daily reference value per serving
	High	6 g per 100 g or 3 g per 100 Kcal or 20 % of daily reference value per serving

Nutrition claims must be written according to national nutrition policies, and only the claims that support these policies should be allowed;

- *health claim* is defined as "any representation that states, suggests, or implies that a relationship exists between a food or a constituent of that food and health". Based on the *Codex Alimentarius* position, health claims are classified in:

  nutrient function claims – they describe the physiological role of a nutrient in development, growth, and normal functions of the body;

  other function claims – they may be used when it is demonstrated that a food constituent or a whole food contribute to the improvement or preservation of health showing a specific positive effect, in health subjects;

  reduction of disease risk claims – they may be used when the consumption of a food constituent or a whole food, in the context of the total diet, lead to a reduction of risk to develop a disease.

  Moreover, also the health claims must be written according to national nutrition policies. Only health claims that support the national policy should be allowed.

Overall, both functional food definitions and claims change among Europe, USA and Japan.

1.1.1 Functional foods in Europe

The attention on the concept of *functional food* has recently developed in Europe, primarily because of the increasing scientific evidences supporting the relationship between health and nutrition. In 1995, following these trends, the Concerted Action on Functional Food Science in Europe (FUFOSE) was promoted. This work was coordinated by the European International Life

Sciences Institute (ILSI Europe), and involved approximately one hundred experts of nutrition, who together elaborated the "Scientific concepts of functional foods in Europe" (1999). This document contain the guidelines for developing a scientific approach to functional foods, and for identifying them. From this intensive action, Diplock et al. (1999) were able to define the concept of functional food, which is still accepted in Europe:

"A food can be regarded as functional if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either improved state of health and well-being and/or reduction of risk of disease. A functional food must remain food and it must demonstrate its effects in amounts that can normally be expected to be consumed in the diet: it is not a pill or a capsule, but part of the normal food pattern".

An ILSI Europe Concise Monograph is downlodable at http://ilsi.org/publication/concepts-of-functional-foods/.

The distinctive features of functional foods are:

- to be consumed as part of the normal diet as "food" (not in pharmaceutical form);
- to have claims demonstrated scientifically (*in vivo* studies; healthy subjects);
- to be made of natural components in concentration to give the beneficial effect;
- to have positive effect on health, beyond basic nutrition, improving the quality of life (physical, behavioural, psychological performances) or reducing the risk of disease.

Therefore, a functional food may be:

- a natural whole food;
- a food enriched in a component;

- a food subtracted in a component;
- a food in which a component has been modified (e.g. to enhance the bioavailability);
- any combination of the above.

A functional food may be designed for the whole population or for particular groups of persons, which may be defined, for example, by age or by genetic constitution (Ghosh et al., 2012).

Many "consensus papers" were published during the last two decades regarding the definition of functional foods, allowing different shades of interpretation (Diplock et al., 1999; Roberfroid, 2002; Roberfroid, 2000). A legislation on functional foods does not yet exist in Europe, but the Regulation (EC) No 1924/2006 on nutrition and health claims was established. It started to apply on 1st of July 2007 and it was amended by Regulation (EU) No 1047/2012. This regulation is a legal framework that food industry operators must use whenever they want to report on the label the beneficial effects (healthy or nutritional) of a certain product.

The purpose of these rules is to ensure that any claim written on a label is accurate, clear, and based on scientific evidences. This European regulation defined nutrition and health claims as following:

A *Nutrition claim* is "any claim which states, suggests or implies that a food has beneficial nutritional properties due to its energy (calorific value) or nutrients". These claims are permitted only if they are listed in the Annex of Regulation (EC) No 1924/2006.

Nutrition claims correlated to these Regulation and the conditions applyed to them, are reported following:

LOW ENERGY

A claim that a food is low in energy, and any claim likely to have the same meaning for the consumer, may only be made where the product does not contain more than 40 kcal (170 kJ)/100 g for solids or more than 20 kcal (80 kJ)/100 ml for liquids. For table-top sweeteners the limit of 4 kcal (17 kJ)/portion, with equivalent sweetening properties to 6 g of sucrose (approximately one teaspoon of sucrose), applies.

ENERGY-REDUCED

A claim that a food is energy-reduced, and any claim likely to have the same meaning for the consumer, may only be made where the energy value is reduced by at least 30 %, with an indication of the characteristic(s) which make(s) the food reduced in its total energy value.

ENERGY-FREE

A claim that a food is energy-free, and any claim likely to have the same meaning for the consumer, may only be made where the product does not contain more than 4 kcal (17 kJ)/100 ml. For table-top sweeteners the limit of 0,4 kcal (1,7 kJ)/portion, with equivalent sweetening properties to 6 g of sucrose (approximately one teaspoon of sucrose), applies.

LOW-FAT

A claim that a food is low in fat, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 3 g of fat per 100 g for solids or 1,5 g of fat per 100ml for liquids (1,8 g of fat per 100 ml for semi-skimmed milk).

FAT-FREE

A claim that a food is fat-free, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0,5 g of fat per 100 g or 100 ml. However, claims expressed as ‘X % fat-free’ shall be prohibited.

LOW-SATURATED FAT

A claim that a food is low in saturated fat, and any claim likely to have the same meaning for the consumer, may only be made if the sum of saturated fatty acids and trans-fatty

acids in the product does not exceed 1,5 g per100 g for solids or 0,75 g/100 ml for liquids and in either case the sum of saturated fatty acids and trans-fatty acids must not provide more than 10 % of energy.

SATURATED FAT-FREE

A claim that a food does not contain saturated fat, and any claim likely to have the same meaning for the consumer, may only be made where the sum of saturated fat and trans-fatty acids does not exceed 0,1 g of saturated fat per 100 g or 100 ml.

LOW SUGAR

A claim that a food is low in sugar, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 5g of sugar per 100 g for solids or 2,5 g of sugar per 100 ml for liquids.

SUGAR-FREE

A claim that a food is sugar-free, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0,5 g of sugar per 100 g or 100 ml.

WITH NO ADDED SUGAR

A claim stating that sugars have not been added to a food, and any claim likely to have the same meaning for the consumer, may only be made where the product does not contain any added mono- or disaccharides or any other food used for its sweetening properties. If sugars are naturally present in the food, the following indication should also appear on the label: ‘contains naturally occurring sugars’.

LOW SODIUM/SALT

A claim that a food is low in sodium/salt, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0,12 g of sodium, or the equivalent value for salt, per 100 g or per 100 ml. For waters, other than natural mineral waters falling within the scope of Directive 80/777/EEC, this value should not exceed 2 mg of sodium per 100 ml.

VERY LOW SODIUM/SALT

A claim that a food is very low in sodium/salt, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0,04 g of sodium, or the equivalent value for salt, per 100 g or per 100 ml. This claim shall not be used for natural mineral waters and other waters.

SODIUM-FREE or SALT-FREE

A claim that a food is sodium-free or salt-free, and any claim likely to have the same meaning for the consumer, may only be made where the product contains no more than 0,005 g of sodium, or the equivalent value for salt, per 100 g.

SOURCE OF FIBRE

A claim that a food is a source of fibre, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 3 g of fibre per 100 g or at least 1,5 g of fibre per 100 kcal.

HIGH FIBRE

A claim that a food is high in fibre, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least 6 g of fibre per 100 g or at least 3 g of fibre per 100 kcal.

SOURCE OF PROTEIN

A claim that a food is a source of protein, and any claim likely to have the same meaning for the consumer, may only be made where at least 12 % of the energy value of the food is provided by protein.

HIGH PROTEIN

A claim that a food is high in protein, and any claim likely to have the same meaning for the consumer, may only be made where at least 20 % of the energy value of the food is provided by protein.

SOURCE OF (NAME OF VITAMIN/S) AND/OR (NAME OF MINERAL/S)

A claim that a food is a source of vitamins and/or minerals, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least a significant amount as defined in the Annex to Directive 90/496/EEC or an amount provided for by derogations granted according to Article 7 of Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods.

HIGH (NAME OF VITAMIN/S) AND/OR (NAME OF MINERAL/S)

A claim that a food is high in vitamins and/or minerals, and any claim likely to have the same meaning for the consumer, may only be made where the product contains at least twice the value of 'source of (name of vitamin/s) and/or (name of mineral/s)'. Contains (name of the nutrient or other substance).

INCREASED (NAME OF THE NUTRIENT)

A claim stating that the content in one or more nutrients, other than vitamins and minerals, has been increased, and any claim likely to have the same meaning for the consumer, may only be made where the product meets the conditions for the claim 'source of' and the increase in content is at least 30 % compared to a similar product.

REDUCED (NAME OF THE NUTRIENT)

A claim stating that the content in one or more nutrients has been reduced, and any claim likely to have the same meaning for the consumer, may only be made where the reduction in content is at least 30 % compared to a similar product, except for micronutrients where a 10 % difference in the reference values as set in Council Directive 90/496/EEC shall be acceptable and for sodium, or the equivalent value for salt, where a 25 % difference shall be acceptable.

LIGHT/LITE

A claim stating that a product is 'light' or 'lite', and any claim likely to have the same meaning for the consumer, shall follow the same conditions as those set for the term 'reduced'; the claim shall also be accompanied by an indication of the characteristic(s) which make(s) the food 'light' or 'lite'.

NATURALLY/NATURAL

Where a food naturally meets the condition(s) laid down in this Annex for the use of a nutritional claim, the term 'naturally/natural' may be used as a prefix to the claim.

Instead, a *health claim* is any claim stating a relationship between food and health. In Europe is the European Food Safety Authority (EFSA) responsible for assessing the scientific evidences of the submitted claims and for their authorization (https://www.efsa.europa.eu/en/topics/topic/nutrition-and-health-claims). Moreover, two types of health claims relevant to functional foods are allowed:

Functional health claims (Article 13 claims) – "They concern specific beneficial effects of the consumption of foods or their constituents, in the context of the total diet on normal functions or biological activities of the body. Such claims relate to a positive contribution to health or to the improvement of a function or to modifying or preserving health". This type of claim makes no reference to a disease or a pathological status. An example of this claim is "*Vitamin D is needed for the normal growth and development of bone in children*".

Moreover, a list of permitted health claims which may be made on foods, other than those referring to the reduction of disease risk and to children's development and health, is reported in European Regulation no. 432/2012, that includes also the conditions of use. An example is "*Beta-glucans contribute to the maintenance of normal blood cholesterol levels*" and the conditions are "*The claim may be used only for food which contains at least 4 g of beta-glucans from oats or barley for each 30 g of available carbohydrates in a quantified portion as part of the meal"*.

Risk reduction claims (Article 14(1)(a) claims) – "Claims relating the consumption of a food or food constituent, in the context of the total diet, to the

reduced risk of developing a disease or health-related condition. Risk reduction means significantly altering a major risk factor(s) for a disease or health-related condition. Diseases have multiple risk factors and altering one of these risk factors may or may not have a beneficial effect". An example of this claim is "*Folate can reduce a woman's risk of having a child with neural tube defects, and sufficient calcium intake may help to reduce the risk of osteoporosis in later life*".

Depending on the lack of precise regulatory issues on "functional foods", health claims are accepted when they refer to the "bioactive ingredients", or to the nutrients/bioactive used to formulate food, also concerning the so called "functional foods".

1.1.2 Functional foods in USA

In USA, like in Europe, functional foods do not have a regulatory identity. However, several definitions have been proposed for functional foods (Hasler, 2002).

"A*ny modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains*" (National Academy of Sciences' Food and Nutrition Board, 1994).

"Foods that, by virtue of the presence of physiologically active components, provide a health benefit beyond basic nutrition" (International Life Sciences Institute).

In a 1999 position paper, the American Dietetic Association defined functional foods as *"whole, fortified, enriched, or enhanced foods, which have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis, at effective levels*" (Thomson et al., 1999).

Moreover, in USA, the term *health claim* indicates any expression of message or representation, including graphic and symbolic, which establishes, suggests or implies that there is a relationship between a certain product or substance and the

ability to reduce the risk of the disease. Therefore, in USA, claims about the reduction of risk disease have been allowed since 1993. They must be authorised by the Food and Drug Administration (FDA) on the basis of "the totality of publicly available scientific evidence and where there is significant scientific agreement amongst qualified experts that the claims are supported by the evidence". Although the operators of food industry may add health claims on their product labels, the aim of health claims, for FDA, should be to benefit consumers by providing information on healthful foods that may help reduce the risk of diseases such as stroke, heart disease and cancer. The FDA announced that claims can also be based on "authoritative statements" of a Federal Scientific Body, such as the National Institutes of Health and Centres for Disease Control and Prevention, as well as from the National Academy of Sciences (Hasler, 2002).

1.1.3 Functional foods in Japan

Japan is the country where the concept of functional food was born. The term FOSHU (*Foods for Specific Health Use)* was created in 1991, and the regulation of these foods was established. The requirements for FOSHU approval are:

- health benefits on the human body must be clear and scientifically proven;
- toxicity must be absent;
- nutritionally suitable ingredients must be used;
- indications on the consumption must be provided;
- information about ingredients, process and methods of analysis must be specified.

The Japanese Institution that approved the functional foods as FOSHU is the Minister of Health and Welfare. A FOSHU can be approved only following revising of the scientific evidences that support the claim and considering that food as part of an ordinary diet (Cheung, 2008).

Figure 1 shows the "logo" of FOSHU, the only one worldwide recognised at National level (conversely, both USA and Europe are missing one).

Figure 1. FOSHU logo

1.2 Nutraceuticals

Another term often used interchangeably with functional foods, although incorrectly, is nutraceuticals. The term *nutraceutical* is a hybrid of *nutrition* and *pharmaceutical*. It was coined by DeFelice in 1989, and indicate *"any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of diseases"* (Andlauer & Furst, 2002). Therefore, the term *nutraceutical* is more generic and quite different from *functional food*, and it is not regulated (Hasler, 2005). Moreover, there are subtle differences between the nutraceuticals and functional foods (Cencic & Chingwaru, 2010):

- nutraceuticals are naturally bioactive compounds that may be found in dietary supplements, foods or botanical products, which promote the health and prevent the diseases;
- functional foods are foods that contain a compound having health-promoting features over the nutritional value of food.

The ingredients used to formulate functional foods and nutraceuticals may be compounds from different sources, those from plant are generally called phytochemicals. The “nutraceuticals” are often consumed in “unit” dose forms such as tablets, pills, capsules or liquids (pharmaceutical forms) and commonly known as food/dietary supplements. The nutraceutical-related offer, beside functional and fortified food sectors, has grown significantly in Europe during the last decade. Moreover, nutraceuticals are largely used to formulate food supplements worldwide. Food supplements are concentrated sources of nutrients (or other substances) with a nutritional or physiological effect. The objective of the harmonised rules on those products in Directive 2002/46/EC (and following amendments) is to protect consumers against potential health risks from those products and to ensure that they are not provided with misleading information. Despite this regulatory framework, a significant problem is correlated to the legal definition of “botanicals” as well as the use of health claims (ingredients from plant origin largely used both for food and food supplements production, requiring more actions for a harmonization at European level.

1.3 Phytochemicals

Phytochemicals are substances from plant sources, and their health properties have underpinned their application as pharmaceuticals and functional food ingredients (Gupta et al., 2015).

Many evidences have highlighted that the consumption of foods rich in phytochemicals, like fruit, cereals, nuts and vegetables, as well as foods enriched in them, is associated with the reduction of risk, and, consequently, with the prevention of the development, of diseases as cardiovascular diseases, stroke, cancer, Alzheimer disease, cataracts, and some of the functional declines

associated with aging (Liu, 2003). However, many convincing evidences suggest that the benefits of phytochemicals in fruit and vegetables are due to their antioxidant activity, which contrast the oxidative stress induced by free radicals and involved in a wide range of deseases, as the chronic ones (Bidlack et al., 2000).

The health properties of fruit and vegetables are attributed also to the complex mixture of phytochemicals; the synergistic effects of phytochemicals in fruit and vegetables are responsible for their potent antioxidant, antinflammatory and anticancer activities (Liu, 2003).

It is also important to consider that phytochemicals are present in fruit and vegetables in amount to give health benefits, but they may be toxic in higher amounts. The right amount depends also by the status and the diet of the subject, therefore there is not a recommended dietary allowance (RDA) for phytochemicals (Liu, 2003).

Phytochemicals vary widely in chemical structure; they are generally classified in carotenoids, isothiocyanates, isoflavones/phytoestrogens and phenolic compounds (Carkeet et al., 2012). The biodiversity of phytochemicals is extremely broad, and often the real positive physiological effect is brought by the "phytocomplex" and not by a single molecule.

1.3.1 Phenolic compounds

The phenols are a class of more than 8000 compounds naturally found in fruits, vegetables, cereals, legumes and nuts (Tsao, 2010). They are secondary metabolites of plants and are generally implicated in defence against the ultraviolet radiation and aggression by pathogens.

Polyphenols comprise a large class of compounds classified into flavonoids, phenolic acids, polyphenolic amides, lignans and stilbenes (Costa et al., 2017; Tsao, 2010).

Many epidemiological in vivo and in vitro studies, as well as meta-analyses, suggest that high polyphenol intake, as in Mediterranean diet, improves human health and can reduce the risk of diseases, like cancer, diabetes mellitus, cardiovascular and neurodegenerative diseases. The main health properties attributed to polyphenols are antioxidant, anti-inflammatory, anticarcinogenic, antiviral and antiallergic effects (Costa et al., 2017).

The polyphenols are found in several foods, including spices and drinks; lycopene, typical of tomatoes, catechins of cocoa and tea, curcumin of *Curcuma longa*, isoflavones of soy, sulforaphane of broccoli and anthocyanins of pomegranate (Diaz-Gerevini et al., 2016; Nam et al., 2016; Venigalla et al., 2016; van Die et al., 2016) were among the most studied polyphenols in the past years. Moreover, polyphenols in foods are susceptible to oxidation, polymerization and other reactions that strongly limit their bioactive properties. The oxidation often occurs along food shelf life, significantly affecting its sensorial properties (e.g. color change).

1.4 Microencapsulation

The development of functional foods often requires the use of technological processes indispensable for the realization of the final product. Particularly, regarding the functional foods enriched with phytochemicals, one of the main problem is related to their inclusion in the food. Phytochemicals are conventionally extracted from the food source using solvents therefore, they are usually in liquid form. Furthermore, many phytochemicals are molecules readily degradable, highly susceptible to processing, environmental and gastrointestinal conditions. Therefore, it is often necessary to apply suitable technologies for their handling and preservation during the technological processes, one of these being encapsulation.

Encapsulation is a physicochemical process of enclosing solids, liquids or gas compounds in an inert shell that isolate and protect the bioactive compound from the environment. When the final product of encapsulation has micron-size dimensions, the process is called microencapsulation (Jyothi et al., 2010).

Microencapsulation is a technological process firstly developed for pharmaceutical purposes to control the release of drug substances, to improve the bioavailability of drugs and to mask the bitter taste of drug substances.

The extension of microencapsulation to the food industry occurred principally to control the release of flavorings and to produce bioactive substances for functional foods. Masking undesirable flavors represents a fundamental strategy in food, for example, when the "functional" ingredient is chacterized by an unpleasant aroma, somehow affecting the palatability.

Microencapsulation can be employed in the food field for different reasons (Gharsallaoui et al., 2007):

- ✓ to protect sensitive and unstable compounds from the surrounding environment;
- ✓ to control or delay the release of a certain compound;
- ✓ to add flavor or to mask the original taste;
- ✓ to dilute the core material;
- ✓ to ease the handling;
- ✓ to enhance the solubility, dispersibility or flowability.

The microencapsulation of substances for food purposes requires the use of *food grade* agents to entrap the core material. This limits the employment of interesting substances, currenlty used in pharma area and not allowed as additives in food area. The core may be composed of one or more ingredients/compounds, while the wall may be single or double-layered. The encapsulated material can go under the name of core, internal phase, or fill, while the wall is sometimes called

shell, coating or wall material. Physically, the core may be a crystalline material, a jagged particle, an emulsion, a suspension of solids, or a suspension of smaller microparticles (Gharsallaoui et al., 2007). The selection of the encapsulation system is, of course, strictly related to the considered food, both lipophily and idrophily behaviours must be considered during the setting up of new encapsulated ingredients. Moreover, color as well as other parameters affecting the texture and the sensorial properties of the final product must be paied particular attention.

There are many microencapsulation techniques usable in food industry (**Fig. 2**), such as spray-drying, coacervation, spray-cooling, spray-chilling, freeze-drying, fluidized bed coating, extrusion-spheronization, coacervation, liposome entrapment, molecular inclusion, and co-crystallization solvent/evaporation (Desai & Park, 2005; Gibbs et al., 1999; Shahidi & Han, 1993).

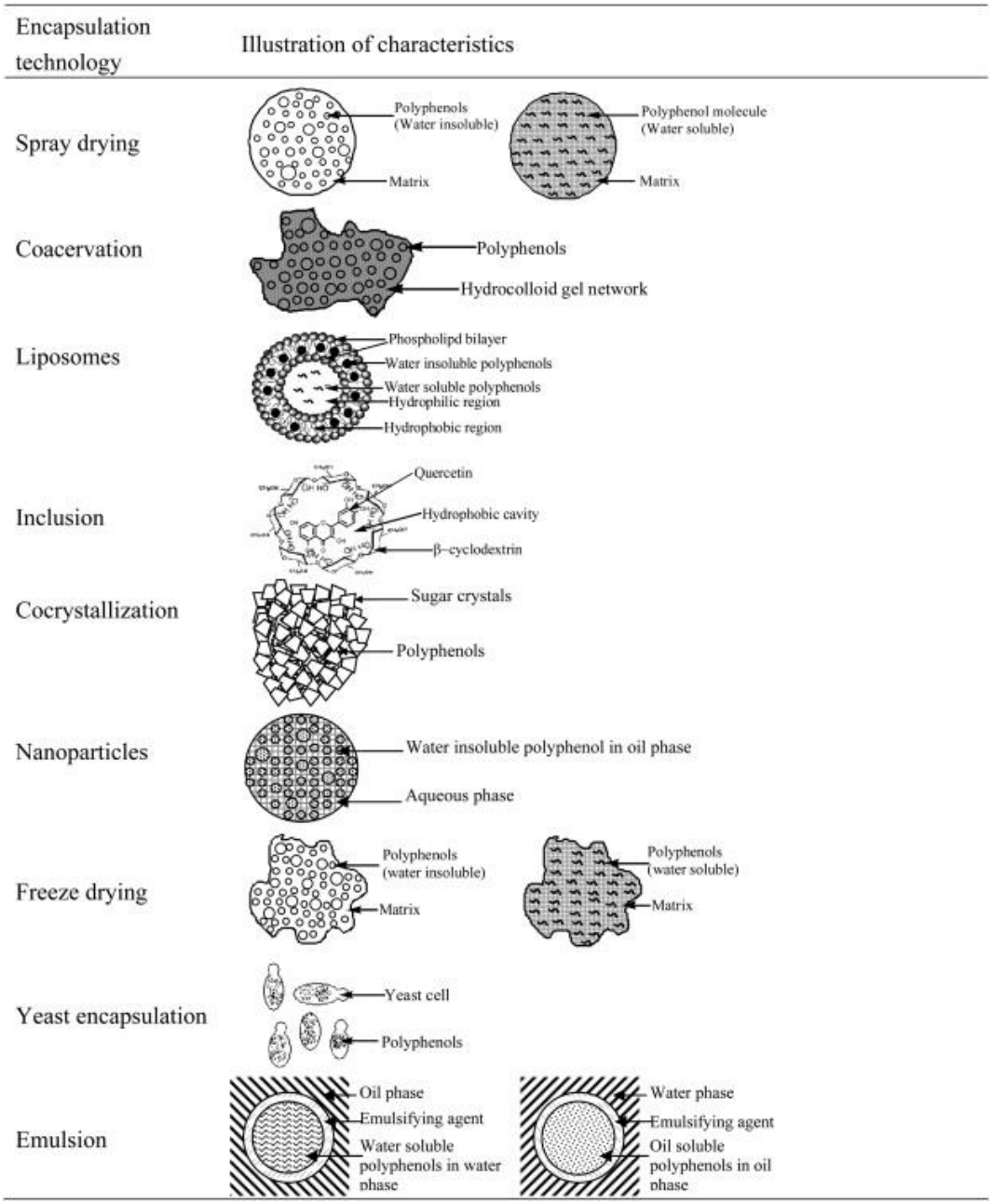

Figure 2. Illustration of encapsulation techniques used for food applications (Fang & Bhandari, 2010).

The choice of microencapsulation technology depends on different factors, as the type of required microparticle, the physicochemical properties of core and shell materials, the efficiency and yield of microencapsulation and, last but not least, the economic aspect (Kuang, Oliveira, & Crean, 2010). An economical evaluation must be performed too, in order to consider the real costs of the process and the benefit of the final food product.

The microencapsulation technique, the physicochemical properties of the core and the wall composition affect the nature of the obtained particle. The microparticles can be 1-1000 μm sized, can have a spherical or an irregular shape and a structure made by one or two layers. The particles are mainly divided in *mononuclear*, when they have only one core coated by a uniform shell, or *matrix type*, when many cores are embedded in a wall matrix (**Fig. 3**) (Fang & Bhandari, 2010).

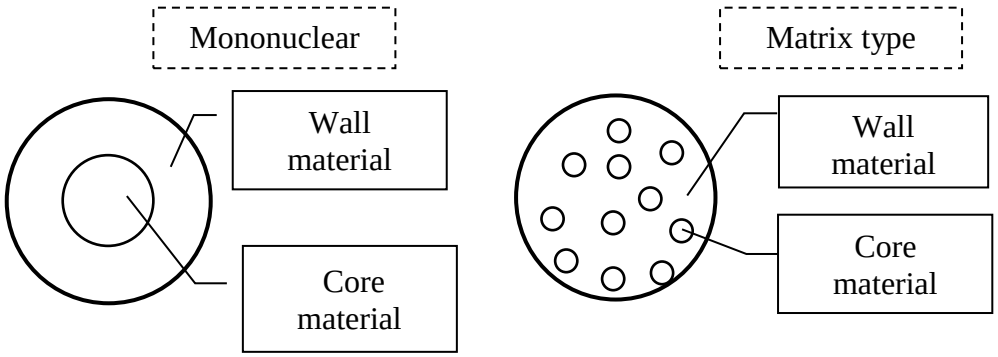

Figure 3. Particles types

Encapsulation by spray drying is largely employed in the food area, and can be performed using many different materials. However, only the substances considered *food grade* and *generally recognized as safe* (GRAS) can be employed to encapsulate bioactive compounds for functional foods. Moreover, the wall material must protect the core material from the environment, as well as have good rheological properties (Schrooyen, van der Meer, & De Kruif, 2001).

The most useful encapsulating materials are carbohydrate-based polymers, especially starch and derivates (amylose, amylopectin, dextrin, maltodextrins), celluloses and derivates (methylcellulose, ethyl cellulose, carboxymethylcellulose, etc), gums (arabic, xanthan, other neutral polysaccharides), pectins, carrageenans and alginate. They are used for their capacity of forming films and their high permeability to water.

Maltodextrins (MD) and gum arabic (GA) are the most suitable wall materials for spray-drying. They are considered 'non sticky' matrices that can be used in many operative conditions to give easily handling powders (Gharsallaoui et al., 2007).

MD are extensively used as wall material in food industry. The reasons are their high water solubility, low viscosity and their colorless solutions (Robert et al., 2010). They are made by chains of D-glucose units linked through α-(1→4) glyosidic bonds, and are produced from starch by partial hydrolysis. MD are typically composed by a mix of chains of variable length, from three to seventeen glucose units long. They are classified by DE (dextrose equivalent), ranging from 2 to 20; high DE values correspond to shorter glucose chains, and the other way around. It was pointed out that MD stabilize the encapsulates from the oxidative insults, but exhibit poor emulsifying capacity (Gharsallaoui et al., 2007). Moreover, they provide thermal protection to different thermolable molecules (e.g phenolic compounds, (Paini et al., 2015), particularly those characterized by high DE values (Schrooyen et al., 2001).

GA is a hydrocolloid produced by the natural exudation of acacia trees (e.g. *Acacia senegal*, *Acacia nilotica*), thus it is also called *Acacia* gum. It is composed by a mix of highly branched polysaccharides (formed by galactose, arabinose, rhamnose, and glucuronic acids) and glycoproteins (Street & Anderson, 1983). The protein component represents the 2% of GA (Renard et al.,, 2012) and plays a crucial role in determining the emulsification properties of GA. It is an efficient

wall material for its high water solubility, low viscosity to high concentration, and ability to emulsify the oil in water (Wang et al., 2011). However, it acts as a semipermeable membrane and shows a limited barrier capacity against oxidation.

Proteins are also used in the food field for their barrier properties, their low permeability to water and oxygen and their great mechanical properties. Commonly employed proteins are derived from milk (α-lactalbumin, β-lactoglobulin, lactoferrin, casein and caseinates, whey protein isolates, bovine serum albumin), or plants (soy protein isolates, wheat proteins, zein). Other proteins from animal origin, like fish gelatin and collagen, are also used.

The lipids suitable to food applications, for their water-insolubility, are fatty acids, waxes (carnauba, candelilla), oils (soy, canola, palm), phospholipids and glycerides (olein, stearin) (Nedovic et al., 2011).

All these food grade materials were largely used in several applications, going from the improvement of compounds delivery during digestions to the thermoprotection.

The solubility of both core and wall material is the most important parameter in microencapsulation. Therefore, another classification distinguishes the wall materials for their water-solubility and their chemical composition (**Fig. 4**).

Moreover, even the processing conditions play a key role in microencapsulation. The temperature, the rate and extent of the spreading, drying, and coalescence of the coating liquid droplets, are the major factors determinating the particle morphology (Werner et al., 2007).

The inlet temperature must be set up on a value higher than the glass transition temperature of wall polymer, so that the polymer is in a flexible form and able to coalesce (Siepmann & Gopferich, 2001; Werner et al., 2007). Therefore, wall polymers with high glass transition temperature are commonly suggested (Williams III & Liu, 2000).

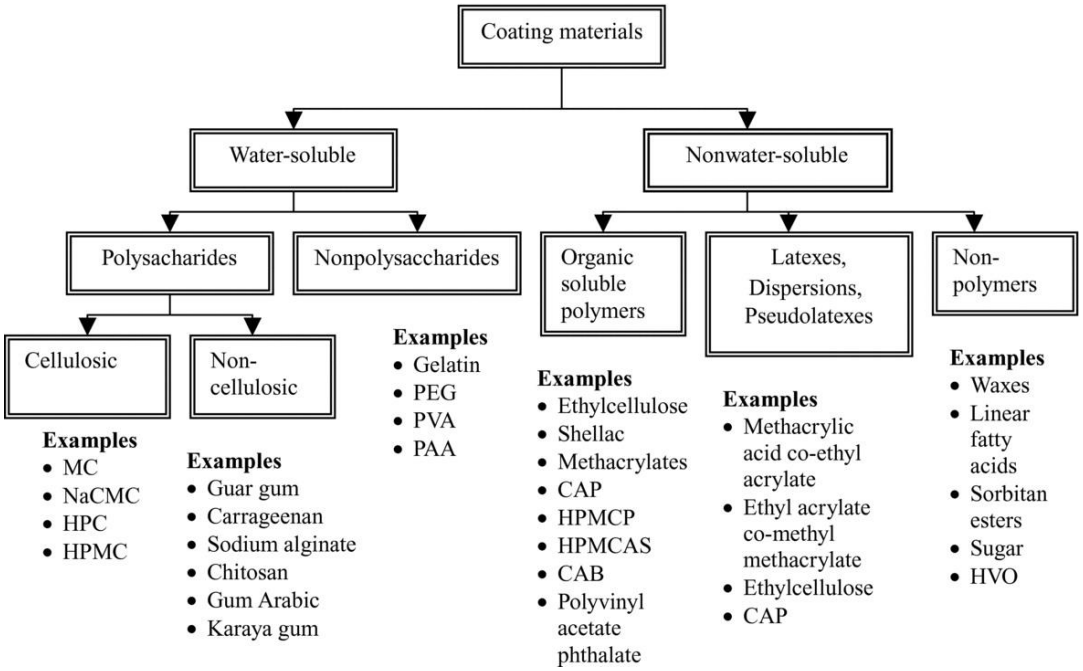

Figure 4. Categories of wall materials. Abbreviations: methylcellulose (MC); sodium carboxylmethyl cellulose (NaCMC); hydroxypropyl cellulose (HPC); hydroxypropylmethyl cellulose (HPMC); polyethylene glycol (PEG); polyvinyl alcohol (PVA); polyacrylic acid (PAA); cellulose acetate phthalate (CAP); hydroxypropyl methylcellulose phthalate (HPMCP); hydroxypropyl methyl cellulose acetate succinate (HPMCAS); cellulose acetate butyrate (CAB); hydrogenated vegetable oil (HVO). (Kuang et al., 2010).

1.4.1 Spray-drying

Spray-drying process has been used since 1950 to encapsulate food ingredients such as fats, flavors, and carotenoids (Gharsallaoui et al., 2007), and in the last years also to incorporate bioactive compounds (vitamins, minerals, omega-3, probiotics, antioxidant compounds, etc.) in foods (Kuang et al., 2010). Among the microencapsulation techniques, spray drying is the most common one and the favourite of food industry, mainly due to its low costs. Even scaling spray drying

up is easier than other techniques, because it is possible to avoid using organic solvents (Kuang et al., 2010).

Microencapsulation by spray drying is carried out by homogeneously dispersing the core material in a solution containing the dissolved wall material, and then by atomizing the mixture into an air stream using a nozzle (**Fig. 5**). The hot air supplies the heat required to remove the solvent, mostly water, from the coating material, thus forming the microencapsulated product that falls to the bottom of the dryer (Gibbs et al., 1999). During this drying process, the evaporation of solvent is rapid, and the entrapment of the interested compound occurs instantaneously (Gharsallaoui et al., 2007), generally giving regular spherical particles ranging 10 to 100 μm of size (Fang & Bhandari, 2010).

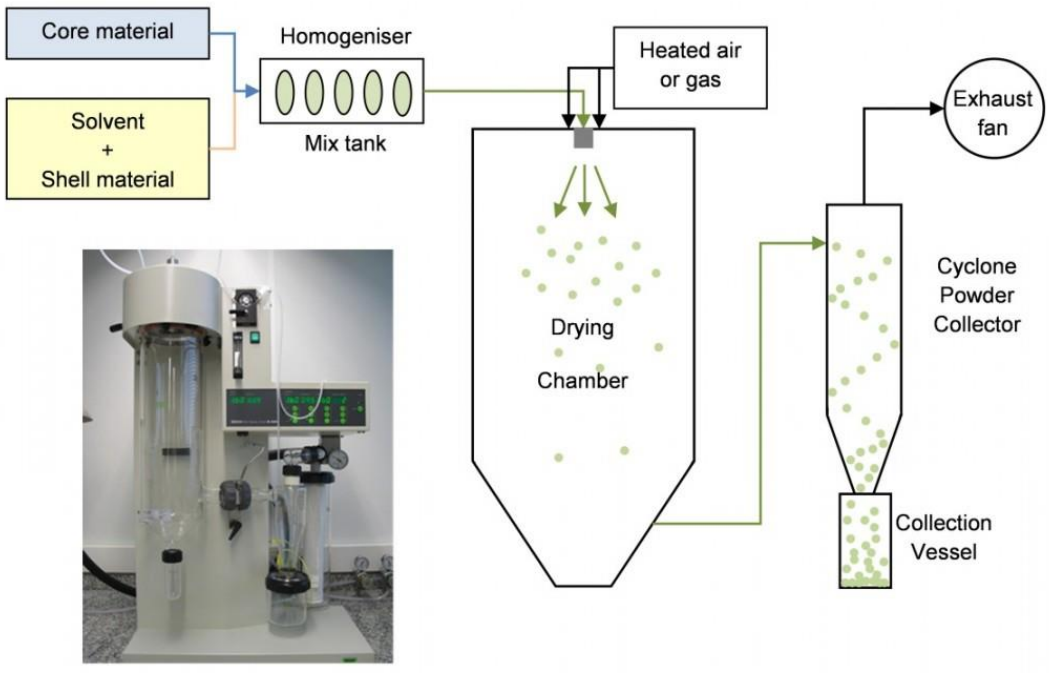

Figure 5. Flow chart of the spray drying process (Chávarri et al., 2012).

Although spray drying is the most employed microencapsulation technique, it presents some limitations, as the low number of wall materials soluble in water at an acceptable level (Desai et al., 2005). Other downsides of spray drying are the complexity of equipment needed, the heterogeneous size of the particles and the high temperature reached during the process. The latter represents a problem for heat labile compounds, like some polyphenols.

In the literature, there are already works discussing how to enhance the stability of polyphenols to the storage, the technological processes and the environmental conditions via encapsulation, as well as to preserve their healthy properties and allow their incorporation into foods.

An example of this kind of application has been reported by Robert et al. (2010), who studied the effects of spray drying on the stability of pomegranate polyphenols. The spray dried powdered polyphenols were added to yogurt to assess their improved stability, as well as to investigate the matrix effect. Moreover, the stability of black currant polyphenols encapsulated with different MD having different DE and inulin, during a storage of 9 month at -20 °C, was assessed by Bakowska-Barczak and Kolodziejczyk (2011). Zhang, Mou, and Du (2007) tested different ratios of wall material and procyanidins to establish the spray dried extract with the longest shelf life, while Paini et al. (2015) analyzed the stability to storage of phenolic compounds from olive pomace spray dried at different inlet temperatures and flow rates. The release rate of bayberry polyphenols encapsulated in ethyl cellulose in the gastro-intestinal tract was assessed by Zheng et al., (2011). Lastly, in other studies, the polyphenols were encapsulated either to reduce their bitter/astringent taste (Gaudette & Pickering, 2013) or to improve their bioavailability (Fang et al., 2010). All these examples confirm a robust proof of concept, showing the usefulness of spray drying polyphenol-based compounds to be added in foods and opening new perspectives for its use in the design of new “functional” ingredients.

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2. Outline of the Thesis

In the last two decades, consumers are increasingly choosing to eat wellness-promoting foods like functional foods, which help to reduce the risk of disease. Among the many factors responsible for this change in attitude towards foods, there are population aging, increased health care costs and new scientific evidences to support the fact that diet can improve health and reduce the risk of catching diseases.

Today, research on functional foods is still in progress and constantly evolving, and it is adding new knowledge on the effects that certain foods/compounds may have on human health.

Many epidemiological, *in vitro* and *in vivo* studies have demonstrated the healthy properties of phytochemicals present in plant foods.

Therefore, one of the aim of food research is to enrich with phytochemicals the foods that are free of or poor in these healthy compounds. The inclusion in foods of polyphenols, a large class of phytochemicals, represents often a challenge for food companies, because they are molecules highly unstable to high temperatures, light, oxygen and enzymes, characteristics that do not consent the application of many processes usually employed in the food sector.

Luckily, the progress of food technologies allowed the development of techniques useful to formulate and stabilize ingredients to be added to foods. One of these is the microencapsulation, a typical pharmaceutical technique: its application in food industry has contributed to the realization of ingredients suitable to these purposes.

Several studies were performed on the microencapsulation of different families of polyphenols, but the contribution of spray drying to the production of ingredients for functional foods is still negligible. Therefore, more investigations are needed to produce bioactive ingredients, such as antioxidant polyphenols, which are stable not only during the process of microencapsulation, but also during all the processes needed to make the final product.

The work reported in this PhD Thesis was focused principally on the formulation and characterization of polyphenol-based ingredients (powdered) added to foods to enrich them in phytochemicals, assessing as well their stability during storage and the bioaccessibility of curcuminoids following encapsulation.

The polyphenols considered in this work belong to different categories, such as flavan-3-ols, anthocyanins and curcuminoids, which are well characterized and recognized as healthy ingredients in many previously published studies.

The first step of the research was optimizing the extraction, using *food grade* solvents, of these compounds from the matrix where they are naturally present.

The second step was to protect and stabilize the extracted bioactive compounds by microencapsulating them through spray drying. Different *food grade* wall materials were tested to formulate powdered polyphenols useful to develop functional food ingredients.

In a third step, model foods, like biscuits, plain yogurt and rice, were enriched with the spray dried ingredients to verify their content in bioactive substances and their antioxidant capacity.

The polyphenols were analyzed and chemically characterized, before and after spray drying, to assess their stability to the process (particularly considering the thermal impact) and over time. The ingredients tested in biscuits were analyzed after the baking to assess their stability to high temperatures, while the ingredient added to yogurt and rice was tested for its gastro-intestinal stability.

In particular, in the first part of the work focused on *Teobroma cacao* polyphenols (obtained from cocoa hulls, a waste of cocoa roasting process), the bioactive fraction was extracted and microencapsulated with different coatings; finally, the powdered polyphenol extracts were added as ingredient to increase the antioxidant capacity of biscuits, used as model food. To evaluate the stability of polyphenols to spray drying, storage and baking, polyphenolic content and antioxidant capacity were spectrophotometrically determined, while the

identification of main flavan3-ols (catechins and procyanidins) and phenolic acids was performed by chromatographic techniques (HPLC-DAD).

A second study, was performed in a similar way on anthocyanins extracted from "Artemide" black rice (*Oryza sativa*). In this work, the polyphenols were also freeze-dried to compare spray drying to a different drying process that does not use high temperatures, to further assess their impact on anthocyanidins. The content of total anthocyanins, polyphenols and condensed tannins, the antioxidant capacity and the amount of each identified anthocyanin, phenolic acid and flavonol was assessed. Also in this case, both spectrophotometric and chromatographic techniques were employed.

In a third study, turmeric (*Curcuma longa*) oleoresin rich in curcuminoids was spray dried, and the obtained powder was added to yogurt and rice. The first aim of this work was to improve the hydrophilic properties of the oleoresin, permitting its dispersion in a water-based medium. To evaluate the stability to the conditions found in the gastro-intestinal tract, both the powder and the enriched yogurt underwent firstly *in vitro* gastro-intestinal digestion (Infogest protocol) and then colonic biotransformation using the simulator of human intestinal microbial ecosystem (SHIME® instrument), which simulated the activity of the three tracts of colon (ascending, transverse and descending). Subsequently, the *in vitro* bioaccessibility of curcuminoids and the matrix effect of rice and yogurt were tested, comparing the microencapsulated ingredient to a sample of turmeric powder, usually employed as spice or food pigment, and to a patented formulation of highly bioavailable curcumin (Phytosome® form, patented and declared able to enhance the bioavailability of curcumin). Part of this third work was performed at the Department of Agrotechnology and Food Sciences of the Wageningen University & Research (Wageningen, The Netherlands).

Chapter 3

Article submitted to Journal of Functional Foods

Microencapsulation of polyphenols from cocoa hulls: a strategy to obtain functional ingredients for bakery products

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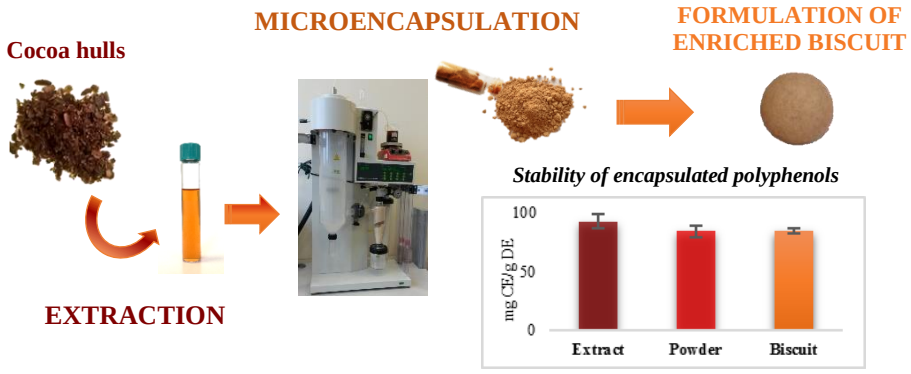
HIGHLIGHTS

- Cocoa hulls are an interesting polyphenol-rich waste
- The polyphenolic profile was characterized by HPLC-DAD
- Microencapsulated cocoa hulls polyphenols are stable over time
- Maltodextrins are useful to obtain powders stable to baking

GRAPHICAL ABSTRACT



ABSTRACT

Cocoa hulls are a potential source of polyphenols to be used as "functional ingredients" in foods, but their low stability to oxidation and thermal degradation limits their practical application. The aim of this study was to microencapsulate cocoa hulls phenolic extracts through spray-drying, in order to produce new heat stable ingredients for bakery products.

Polyphenols were extracted using water and ethanol under different conditions. The best performing extract (water/ethanol 50:50) was spray-dried with and without stabilizing agents (maltodextrins and/or gum arabic), obtaining seven different powders. These were first tested for their stability over time, showing a total phenolic content and an antioxidant capacity stable up to 90 days. The powders were then used to evaluate their baking stability in a model biscuit; the microencapsulation using an 80:20 ratio of maltodextrins to the dry extract allowed obtaining the most stable powder, with a total polyphenol content unaffected by the baking process.

KEYWORDS

Spray-drying, antioxidant capacity, HPLC, waste, functional foods, thermo-protection

1. INTRODUCTION

Theobroma cacao L. and its derived products are food matrices rich in polyphenols, mainly represented by flavanols (Miller et al., 2006). Increasing evidences suggest and support that a regular consumption of cocoa may trigger beneficial effects, especially with regard to cardiovascular diseases, metabolic disorders, and cancer prevention (Andujar, Recio, Giner, & Rios, 2012; Corti, Flammer, Hollenberg, & Luscher, 2009). Many *in vitro* and *in vivo* studies were performed in order to correlate the polyphenols and the beneficial effects of cocoa, primarily antioxidant and anti-inflammatory properties (Donovan, Holes-Lewis, Chavin, & Egan, 2011; Ellam & Williamson, 2013). Therefore, the European Food Safety Agency (EFSA) has confirmed the beneficial properties of cocoa on health, permitting the use of the health claim "cocoa flavanols help maintain the elasticity of blood vessels, which contributes to normal blood flow" (EFSA, 2012).

The main polyphenols present in cocoa beans belong to the class of flavan-3-ols, the 34-37% of which are represented by the monomeric forms (Wollgast & Anklam, 2000): (-)-epicatechin is the most abundant component, (+)-catechin is present in smaller amounts, whereas (+)-gallocatechin and (-)-epigallocatechin can be found in traces. Polymeric flavanols, the procyanidins, are the most abundant flavanols of bean (58%), and are mainly bound to the fiber (Lecumberri et al., 2007). Cocoa procyanidins are polymers with a degree of polymerization even higher than 10 (Gu et al., 2002), the most abundant is dimer B2 [epicatechin-(4 β→8)-epicatechin].

Processing of cocoa beans allows substantial changes in their composition, leading even to the modification of the bioactive properties (Wollgast & Anklam, 2000). More particularly, the roasting process, required to develop the Maillard Reactions (MRs) and to create the typical "cocoa aroma", reduces the antioxidant capacity (Oliviero, Capuano, Cammerer, & Fogliano, 2009). Moreover, the

processing of the cocoa beans leads to the production of large quantities of cocoa hulls (CH), currently a waste considered source of interesting bioactive compounds (Okiyama, Navarro, & Rodrigues, 2017).

CH represent the 9.8% of beans (Ntiamoah & Afrane, 2008), and are rich in fiber and polyphenols, which composition depends on the variety, the country of origin, the fermentation and processing operations (Oracz, Zyzelewicz, & Nebesny, 2015; Bordiga et al., 2015). Considering that CH are promising sources of polyphenols and a major disposal problem for the cocoa industry, the recovery of these bioactive compounds may be useful in the formulation of *functional foods* enriched in antioxidant compounds (Makris, Boskou, & Andrikopoulos, 2007), especially in flavanols.

The methodologies to extract exhaustively phenolic compounds generally employ organic solvents, but often the most performing solvents cannot be used in food industry. Alternative strategies (food-grade solvents, extractions with ultrasounds, microwave, subcritical water and supercritical fluids) could be a solution, also in order to minimize the environmental impact, although many of these are hardly scalable at industrial level (Vilkhu, Mawson, Simons, & Bates, 2008).

The direct addiction of polyphenol extracts in cooked foods is not possible, due to the easy thermal degradation/oxidation of polyphenolic compounds (Zheng, Ding, Zhang, & Sun, 2011). A solution might be the microencapsulation, a technology able to entrap a substance in a coating agent in order to produce encapsulated particles of few micrometers protecting the substance from the outside environment (Dias, Ferreira, & Barreiro, 2015).

The microencapsulation technology most commonly used in food industry, for its cheapness, usefulness, and scale ability, is spray-drying. Spray dried powders show a low water activity, which limits or even prevents the degradation, preserves the biochemical functionalities of the components, reduces storage and

transport costs, enhances the instantaneous solubility of the final product and improves the microbiological stability (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007).

Spray-drying allows to obtain "matrix type" microspheres (Zuidam & Shimoni, 2010), where the active ingredient is dispersed in the stabilizing agent (SA), which forms a barrier between the ingredient and the environment.

The SAs used in food industry are usually polymeric carbohydrates, with good interfacial properties, selected both in accordance with the chemical-physical properties of the active ingredient and with the purpose of the microencapsulation (inhibition of the oxidative reactions; masking of flavors, colors and odors; achievement of a sustained/controlled release).

As previously reported, some natural antioxidant compounds are preserved by SAs, like the maltodextrins (MD) (Schrooyen, van der Meer, & De Kruif, 2001), able to partially protect phenolic compounds from thermal degradation. Even the gum arabic (GA), acting also as an emulsifier (Paini et al., 2015; Randall, Phillips, & Williams, 1988) and film forming agent (Madene, Jacquot, Scher, & Desobry, 2006), can be used for this purpose. In several cases, mixtures of MD and GA were used to improve the final result (Zhang, Mou, & Du, 2007).

Several studies were performed on the microencapsulation of different antioxidant extracts (Fazaeli, Emam-Djomeh, Ashtari, & Omid, 2012; Robert et al., 2010; Tuyen, Nguyen, & Roach, 2010), but no one was focused on the stabilization of polyphenols from CH used as ingredients in baked foods. Therefore, the aim of this study was to microencapsulate CH polyphenols to improve their stability over time and during baking. CH polyphenols were spray-dried with MD and GA in different ratios, and then added as functional ingredients to enrich model biscuits. All the samples were characterized for the polyphenolic composition and the antioxidant capacity.

2 MATERIAL AND METHODS

2.1 Materials

CH was kindly provided by an Italian company. They were finely ground with a Tecator Cyclotec 1093 (International PBI, Milan, Italy) laboratory mill equipped with a 500-μm sieve.

Ethanol, methanol, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), GA, and polyphenol standards [(±)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2, gallic acid, protocatechuic acid and *p*-OH-benzoic acid] were purchased from Sigma Aldrich (St. Louis, MO, USA), while MD (dextrose equivalent 16-20%) from A.C.E.F. (Fiorenzuola d'Arda, Italy). Standard solutions were prepared with methanol and stored in dark bottles at -20 °C.

2.2 Extraction of polyphenols from CH

Polyphenols were extracted from CH following different protocols, as summarized in **Table 1**. Water and a mixture of water and ethanol (50:50 v/v) were used as *food grade* solvents at two different ratios with sample (10:1 and 10:2 mL/g). Magnetic stirring and/or ultrasonic agitation were applied.

The extracts were centrifuged at 2800 *x g* for 5 min at 4 °C, then the supernatants were filtered through a Whatman No.1 filter paper (Whatman International Ltd., Maidstone, England). The ethanol was successively removed by a rotary evaporator (*Rotavapor® Büchi R-210*) to avoid the precipitation of SAs in the next step. The extracts were finally stored at -20 °C.

Table 1 Extraction methods (A-H) applied to CH. M= magnetic stirring; S=sonication. The dry extract (DE) was determined by drying the extracts in oven at 110 °C, overnight.

	Solvent	V (mL)	sample (g)	Agitation technique	Time (min)
A	water	10	1	M	30
B	water	10	2	M	30
C	water	10	1	M+S	15+15
D	water	10	2	M+S	15+15
E	water	10	1	S	30
F	water	10	2	S	30
G	water/ethanol	10	1	M+S	15+15
H	water/ethanol	10	2	M+S	15+15

2.3 Spray-drying of the polyphenolic extract

The extract characterized by the highest total phenolic content was selected for the microencapsulation. Seven formulations (from now on referred as powders) were prepared with different MD/GA/DE ratios, in order to obtain powdered extracts (P1-P7) with different compositions, as reported in **Table 2**.

The SA was added directly to the extract, mixed with a constant stirring at 1000 *rpm* for 10 min and then treated with a high-performance disperser (Ultra-Turrax®, T 25 basic Ika®-Werke GmbH, Staufen, Germany) at 13500 *rpm* for 2 min.

Different blends were spray-dried using the *Mini Spray Dryer B-290 (*Büchi®, Switzerland) at inlet temperature of 150 °C, and with a feed flow and airflow of 7 mL/min and 40 m^3/h, respectively.

Table 2 Composition of the powders (P1-P7). P1 extract was spray-dried without SA (control). P2-P6 had SA:DE ratio of 80:20 (w/w), P7 of 60:40 (w/w).

	P1	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**
% MD/GA/DE (w/w/w)	0/0/100	80/0/20	64/16/20	40/40/20	16/64/20	0/80/20	60/0/40

2.4 Extraction of polyphenols from powders

The powders were stored for three months at -20 °C and analyzed every thirty days, in order to evaluate the stability to oxidation over time. Polyphenols were extracted from the powders following the method described by Zhang et al. (2007), with some modifications. Thirty milligrams of powder were extracted firstly with 1.5 mL of water, mixed by vortex for 1 min and sonicated for 10 min. The sample was then centrifuged at 20800 *x g* for 5 min at 4 °C, and the supernatant was recovered. The pellet was dissolved adding 1.5 mL of hydroalcoholic solution (methanol/water, 80:20, V:V), agitated for 1 min and sonicated for 20 min, then centrifuged at 2800 *x g* for 5 min at 4 °C. The two resulting supernatants were employed separately for the downstream analyses. Each powder was extracted in triplicate.

2.5 Formulation of the model food (biscuit)

The seven powders were used separately to formulate and bake biscuits (named from B1 to B7, like the corresponding powders), following the AACC method (Gaines, 1986) with some modifications. The ingredients used were: wheat flour (55.2%), sugar (13.7%), shortening (29.5%) and an amount of powder as to reach the 0.32% of DE in the crude biscuit. Moreover, a control biscuit was prepared only with flour, sugar and shortening, without the addition of polyphenols. The

flour and the powders were firstly mixed homogeneously with a pin mixer; the sugar and the shortening were mixed for 1 min, combined to the flour and mixed for 5 min. The dough was lied with a thickness of 0.7 mm and a circular mold of 4.5 cm was used to give shape to the biscuits. The biscuits were cooked in conventional ventilated oven for 25 min at 180 °C.

2.6 Extraction of polyphenols from model biscuits

In order to determinate the polyphenol stability after baking, biscuits were finely ground and defatted. Lipid fraction was extracted for 6 h using a semiautomatic Soxhlet extraction system B-811 (Büchi, Switzerland), employing dichloromethane as organic solvent.

One gram of each defatted biscuit was extracted twice: firstly, 2 mL of water were added, suspensions were vortexed for 1 min and sonicated for 10 min. Samples were then centrifuged at 15000 *x g* for 2 min at 4 °C, and the aqueous supernatants were recovered for further purification. Pellets were instead dissolved in 2 mL of methanol, mixed for 1 min and sonicated for 10 min, then centrifuged at 15000 *x g* for 2 min at 4 °C. The resulting supernatants were directly analyzed.

Previously obtained aqueous supernatants were purified and concentrated using a C-18 SPE cartridge (SupelCleanTM LC-18 SPE Tubes bed wt, 500 mg; volume, 3 mL, Supelco, Bellefonte, Pa., U.S.A.). Each cartridge was placed in a vacuum elution apparatus and firstly conditioned by loading consecutive volumes of methanol and water. The extracts were loaded into the column and washed by HPLC-grade water. Finally, the column was eluted with 2 mL of methanol and the collected fraction analyzed. Each biscuit was extracted in triplicate.

2.7 Analytical methods

2.7.1 Total Polyphenols

Total polyphenols (TP) content was assessed on CH, powders' and biscuits' extracts, using a modified version of the Folin–Ciocalteu's method (Bordiga et al., 2015). One hundred microliters of Folin–Ciocalteu's reagent (Sigma-Aldrich) and 350 µL of aqueous Na_2CO_3 (5% w/v) were added to the extracts. The solutions were then diluted with water to a final volume of 2.9 mL. The absorbance was read at 760 nm after one hour, using an Evolution 60S spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). Results were expressed as catechin equivalents (CE) through a calibration curve. For powders' and biscuits' extracts, results were expressed as the sum of the quantification carried out on both aqueous and methanolic fractions.

2.7.2 Antioxidant capacity

Antioxidant capacity (AOC) was assessed on CH, powders' and biscuits' extracts, using the DPPH• assay according to the method described by Locatelli et al. (2009). Briefly, 700 µL of opportunely diluted sample or methanol (control) was added to the same volume of a 100 µM DPPH• methanolic solution. This solution was shaken vigorously and left in the dark at room temperature for 20 min, after which the absorbance was read at 515 nm. The AOC was expressed as Trolox equivalents (TE) through a calibration curve. For powders' and biscuits' extracts, results were expressed as the sum of the quantification carried out on both aqueous and methanolic fractions.

2.7.3 RP-HPLC/DAD analysis

The identification and quantification of polyphenols was assessed on CH, powders' and biscuits' extracts following the procedure described by Bordiga et al. (2015). A Shimadzu LC-20A Prominence chromatographic system equipped

with a diode array detector (DAD detector SPD-M20A) was employed to analyze the main cocoa polyphenols: (+)-catechin, (-)-epicatechin, procyanidin B1, procyanidin B2, gallic acid, protocatechuic acid and *p*-OH-benzoic acid. Separation was performed on a reversed-phase Ascentis RP Amide column (150 mm x 2.1 mm; Supelco, Bellefonte, PA, USA). The eluents were: (A) acidified water (0.1% formic acid) and (B) acidified acetonitrile (0.1% formic acid). The gradient program was: 2.5% B (0.1-10 min), 12% B (35 min), 100% B (66 min), 2.5% B (68-78 min), at a constant flow rate of 0.4 mL/min and a temperature of 30 °C. All compounds were identified comparing retention time and UV/Vis spectra of each respective standard. Calibration curves of each compound (at 280 nm) at six different concentration levels, were used for the quantification, except for procyanidin B1 and B2, which were quantified as (-)-epicatechin equivalents. For powders' and biscuits' extracts, results were expressed as the sum of the quantification carried out on both aqueous and methanolic fractions.

2.8 Morphological characterization of microspheres

The powder P2 was morphologically characterized, in order to establish size, shape and surface features. Both size and shape of P2 microspheres were obtained by the Morphologi G3 system (Malvern Instruments, Worcestershire, United Kingdom), based on the automated image analysis, according to a Standard Operating Procedure (SOP) and applying a post analysis filter to remove images of touching particles from the final results. They were analyzed in terms of diameter, circularity and elongation according to Ulusoy and Kursun (2011). The surface morphology of the same sample was carried out by a scanning electron microscopy (SEM) Phenom XL (Phenom-World, Eindhoven, Netherlands), equipped with two detector systems: the first one is a four-segment BackScatter Detector, fully integrated EDS system; the second one is a Secondary Electron

Detector (SED). Samples of microspheres were placed directly on a SEM stubs "charge reduction".

2.9 Statistical analysis

Results were reported as mean ± standard deviation of at least three experiments. The significance of differences was determined by ANOVA, followed by Tukey's post hoc test ($p < 0.05$) using XLStat 365 (Addinsoft, Paris, France).

3 RESULTS AND DISCUSSION

3.1 Characterization of the extracts

The CH extracts, obtained as summarized in **Table 1**, were analyzed for their polyphenolic profile, focusing on the determination of TP, AOC and on the characterization of individual major polyphenols. These analyses allowed to select the best extract, in terms of polyphenols concentration, to be microencapsulated and used as functional ingredient in biscuits.

3.1.1 Total polyphenols content and antioxidant capacity

The TP and AOC values of extracts obtained as described in Table 1 are reported in **Figure 1.**

AOC values ranged from 69.5 to 215 mg of TE per gram of DE for extracts B and G, while TP values ranged from 40.8 to 93.3 mg of CE per gram of DE for extracts F and G, respectively. The use of a solvent/sample ratio of 10:1 led to a greater AOC when compared to the same extraction performed with a ratio of 10:2 (A>B, C>D, E>F and G>H) ($p<0.05$). On the contrary, for the TP content the only significant difference ($p<0.05$) was registered when the extraction was

performed using either water or ethanol (G>H). Overall, extract G, obtained from 1 g of CH in 10 mL of water and ethanol (50:50) stirred for 15 minutes and sonicated for other 15 minutes, showed the highest TP content and AOC, when compared to the samples extracted only by water. Extract G showed a TP concentration of 1.1% (w/w), similar to that reported in Arlorio, Coisson, Restani, and Martelli (2001), which was 1.8% (w/w).

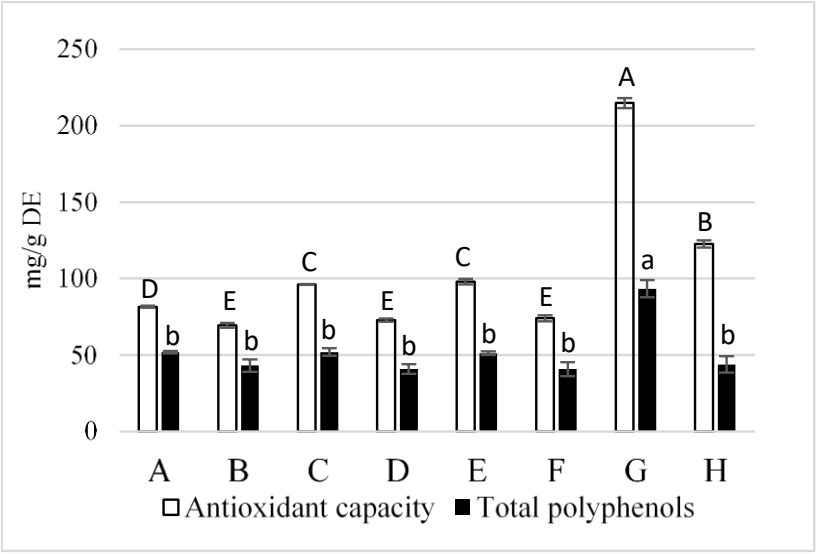


Figure 1 AOC and TP of CH extracts (A-H). AOC is expressed as mg TE/g DE. TP is expressed as mg CE/g DE. Different capital letters indicate significant differences for the AOC values ($p < 0.05$). Different lowercase letters indicate significant differences for the TP content ($p < 0.05$).

3.1.2 Characterization of polyphenolic profile

The extracts obtained using a solvent to sample ratio of 10:1 (w/w), characterized by TP content and AOC higher than the corresponding extracts at 10:2 ratio, were characterized for their polyphenol profile by HPLC-DAD. **Table 3** shows the quantification of the main flavanols ((+)-catechin, (-)-epicatechin,

procyanidin B1 and procyanidin B2) and phenolic acids (gallic acid, protocatechuic acid and *p*-OH-benzoic acid) identified in these samples.

The HPLC data confirmed that the extract G was the richest in terms of polyphenols content. This sample showed the highest concentration of flavanols: epicatechin (2.10 mg/g DE) and catechin (1.05 mg/g DE) were about 4 times higher when compared to other extracts. These results confirmed that epicatechin is the most abundant monomeric flavanol both in CH and in cocoa beans (Wollgast & Anklam, 2000). Moreover, the amount of epicatechin registered in the CH extracted with ethanol and water (extract G) was 25 mg per 100 grams of hulls, about half of what observed in dark chocolate (Bordiga et al., 2015).

The presence of ethanol in extract G also improved the extraction of procyanidins, in particular of procyanidin B2, which increased of 17% when compared to extracts obtained using only water. The procyanidin B1 was more concentrated than procyanidin B2 and a similar result was observed by aqueous enzymatic extraction (Ramos et al., 2008).

The main phenolic acid recovered in these samples was the protocatechuic acid, ranging from 1.33 to 2.18 mg/g of DE in extracts A and G, while benzoic and gallic acids were present in minor amounts, ranging from 0.41 to 0.56 and from 0.22 to 0.40 mg for gram of DE, respectively.

Thus, also considering the concentration of individual compounds, the extract "G" (water/ethanol, 50/50 v/v), resulted the richest in polyphenols, and consequently was selected for the microencapsulation. This extract was then spray-dried with different SAs, obtaining the 7 powders reported in Table 2.

Table 3 Main flavanols and phenolic acids of CH extracts (A, C, E, G). Data are expressed as mg/g DE. Different letters indicate significant differences among the extracts (within each column) ($p < 0.05$).

	Epicatechin	Catechin	Procyanidin B1	Procyanidin B2	Protocatechuic acid	*p*-OH-benzoic acid	Gallic acid
A	0.50 ± 0.05 b	0.21 ± 0.01 b	0.55 ± 0.01 b	0.23 ± 0.04 c	1.33 ± 0.41 d	0.41 ± 0.00 b	0.22 ± 0.01 c
C	0.54 ± 0.05 b	0.23 ± 0.02 b	0.64 ± 0.10 b	0.30 ± 0.03 b	1.47 ± 0.52 c	0.52 ± 0.02 a	0.35 ± 0.01 b
E	0.56 ± 0.05 b	0.24 ± 0.03 b	0.68 ± 0.12 ab	0.28 ± 0.02 bc	1.67 ± 0.55 b	0.55 ± 0.02 a	0.34 ± 0.01 b
G	2.10 ± 0.03 a	1.05 ± 0.01 a	0.83 ± 0.03 a	0.90 ± 0.02 a	2.18 ± 0.02 a	0.56 ± 0.02 a	0.40 ± 0.01 a

3.2 Characterization of powders and biscuits

3.2.1 Powders stability: determination of TP and AOC

TP content and AOC of the 7 powders were determined both at t_0 and during the storage, in order to evaluate the impact of the spray drying process and the stability of the powder over time (**Tab. 4).**

Comparing the values of TP content and AOC of extract G before (**Fig. 1**) and after spray-drying (**Tab. 4,** column t_0), we can say that the process did not significantly affect the AOC, while the TP content remained constant only in P2, P3, P5 and P6. As expected, the lowest TP value (79.8 mg CE/g DE) was registered for P1 (the powder spray-dried without the SA). Although for P1, P4 and P7 spray-drying decreased the TP content of the hydroalcoholic extract, the microencapsulated powders were not significantly different at t_0.

The TP content of the single powders did not decrease significantly ($p<0.05$) also during the 90 days, except for P6 (prepared using 100% GA coating) and P7 (characterized by a different SA/DE ratio of 60/40) powders, which saw a decrease of 17 and 14%, respectively. Significant differences were not

highlighted in samples considered from t_{30} to t_{90}; after 90 days of storage, TP values ranged from 70.8 to 79.1 mg of CE per gram of DE for P7 and P5, respectively.

Unlike the TP, the AOC values of each powder significantly decreased over time ($p<0.05$), except for P1 and P3, which showed a similar AOC during the 90 days. A significant decrease of about 14% was observed during the first thirty days in the AOC of P2, P4, P5, P6 and P7, while from t_{30} to t_{90} it remained constant. Nevertheless, no significant differences were registered comparing the AOC values of different powders when evaluated at the same time.

Finally, all the powders showed similar TP content and AOC after 90 days of storage. Likewise, Bakowska-Barczak and Kolodziejczyk (2011) did not register significant decreases of TP content and AOC in blackcurrant extracts spray-dried with MD during 9 months storage.

Table 4 TP and AOC of the powders (P1-P7) measured at t_0, t_{30}, t_{60} and t_{90}. Different lowercase letters represent significant differences within each row ($p < 0.05$). Different capital letters represent significant differences within each column ($p < 0.05$). In brackets are reported the percent variations of the powder at t_0 compared to the extract G, when there is significant difference ($p<0.05$).

TP (mg CE/g DE ± SD)

	t_0	t_{30}	t_{60}	t_{90}
P1	79.8 ± 2.7 ^{aB} (-16%)	76.9 ± 1.2 ^{aA}	77.2 ± 0.7 ^{aAB}	74.0 ± 2.2 ^{aA}
P2	84.9 ± 4.8 ^{aAB}	77.7 ± 3.1 ^{aA}	76.4 ± 1.0 ^{aAB}	74.5 ± 3.3 ^{aA}
P3	85.6 ± 1.7 ^{aAB}	83.4 ± 5.0 ^{aA}	85.9 ± 2.0 ^{aA}	78.0 ± 5.8 ^{aA}
P4	83.2 ± 1.4 ^{aAB} (-12%)	78.6 ± 0.1 ^{aA}	78.9 ± 2.4 ^{aAB}	79.1 ± 0.7 ^{aA}
P5	87.6 ± 5.0 ^{aAB}	85.0 ± 3.2 ^{aA}	85.5 ± 5.4 ^{aA}	79.1 ± 3.3 ^{aA}
P6	89.8 ± 1.1 ^{aA}	79.9 ± 4.5 ^{abA}	80.6 ± 1.5 ^{abAB}	74.8 ± 4.9 ^{bA}
P7	82.5 ± 3.8 ^{aAB} (-13%)	71.5 ± 4.2 ^{abA}	71.2 ± 0.6 ^{abB}	70.8 ± 3.4 ^{bA}

AOC (mg TE/g DE ± SD)

	t_0	t_{30}	t_{60}	t_{90}
P1	187 ± 14 ^{aA}	171 ± 2 ^{aA}	171 ± 1 ^{aA}	167 ± 1 ^{aA}
P2	199 ± 17 ^{aA}	167 ± 2 ^{bA}	168 ± 7 ^{bA}	166 ± 2 ^{bA}
P3	193 ± 15 ^{aA}	187 ± 13 ^{aA}	184 ± 4 ^{aA}	183 ± 8 ^{aA}
P4	210 ± 3 ^{aA}	186 ± 1 ^{abA}	184 ± 14 ^{bA}	179 ± 16 ^{bA}
P5	215 ± 12 ^{aA}	184 ± 2 ^{bA}	176 ± 3 ^{bA}	176 ± 1 ^{bA}
P6	213 ± 5 ^{aA}	176 ± 8 ^{bA}	182 ± 1 ^{bA}	178 ± 12 ^{bA}
P7	191 ± 4 ^{aA}	165 ± 10 ^{bA}	162 ± 1 ^{bA}	162 ± 3 ^{bA}

3.2.2 Evaluation of baked products: TP content and AOC

In order to study the stability to baking of the different powders, they were added as ingredients in 7 model biscuits, and the TP content and AOC were determined after cooking the biscuits in oven at 150 °C. The results, reported in **Figure 2**, were calculated by subtracting the contribution of the control biscuit (prepared without CH extract), and expressed per gram of DE.

TP content and AOC in biscuits ranged from 28.0 to 84.6 mg CE/g DE, and from 53.9 to 108 mg of TE/g DE, respectively; the lowest value was obtained for the biscuit prepared from the powder without stabilizing agent (B1), while the highest value was registered for the biscuit obtained from the powder with only MD (B2).

The AOC after baking, respect to the corresponding powders measured at t_0, significantly decreased ($p<0.05$) in all the biscuits (from B1 to B7, of 71, 46, 52, 66, 61, 62 and 48%, respectively). The TP content of B2 and B3 remained instead unaltered ($p<0.05$) after baking, while it significantly decreased in all other biscuits. Therefore, B2 and B3, obtained from P2 (MD, 100%) and P3 (MD/GA, 80:20 w/w), showed the highest AOC and TP content.

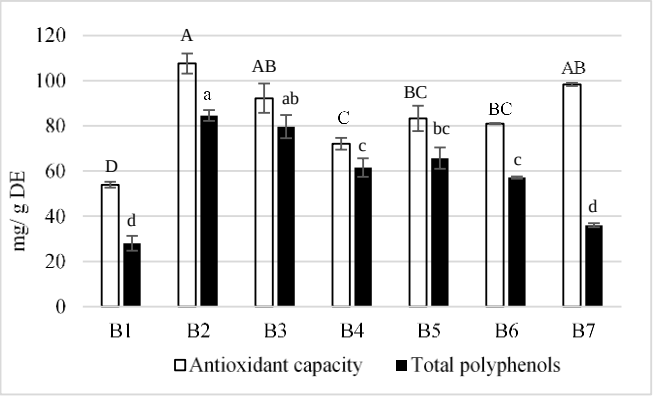


Figure 2 AOC and TP after baking (B1-B7). AOC is expressed as mg TE/g DE. TP is expressed as mg CE/g DE. Different capital letters indicate significant differences for the AOC values ($p < 0.05$). Different lowercase letters indicate significant differences for the TP content ($p < 0.05$).

3.2.3 HPLC analysis of polyphenols in powders and biscuits

The polyphenolic profile of the powders (**Fig. 3**), before and after the baking (biscuits), was characterized by HPLC-DAD.

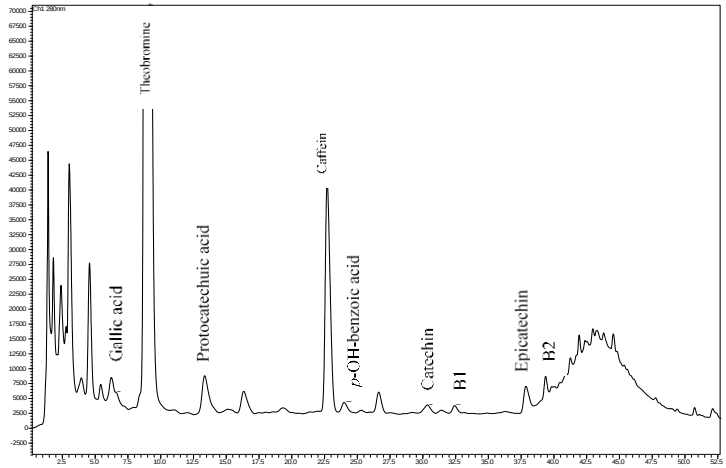


Figure 3 HPLC/DAD chromatogram of the powders

The quantification of the main identified compounds in the powders, expressed as mg/g DE, is reported in **Table 5**; in the last column, the recovery of polyphenols (expressed as percentage) after the spray-drying process (powders), is also reported. The spray-drying process did not overly affect the polyphenolic profile of the powders, which showed values similar to those of extract G for epicatechin, procyanidins B1 and B2, and gallic acid. The procyanidin stability to this process was previously studied by Zhang et al. (2007), who confirmed that no changes occurred in the composition of procyanidins from grape seeds after spray-drying with MD/GA (60:40 w/w) at inlet temperature of 190 °C. On the contrary, a significant decrease was observed for catechin, protocatechuic acid and p-OH-benzoic acid, even though to different extents, depending on the molecules and powders considered. The recovery following spray-drying was greater (94%) for the extract microencapsulated with 80% MD and 20% GA (P5) and lower (82%) for the powder without SA (P1), while for the other five powders ranged from 88 to 89%. These results were in line with those obtained by spectrophotometry.

Table 5 Characterization of main CH polyphenols recovered following spray-drying (P1-P7). Data are expressed as mg/g DE. Different letters indicate significant differences within each column ($p < 0.05$). In brackets are reported the percent variations of each compound in the powder compared to the extract G, when there is a significant difference ($p<0.05$). Recovery values of the powders are calculated by comparing the sum of each compounds in the powder to the corresponding value of the extract G (cfr **Table 3**).

	Monomeric flavanols		**Procyanidins**		**Phenolic acids**			**Recovery (%)** ***(RSD %)***
	Epicatechin	**Catechin**	**B1**	**B2**	**Protocatechuic**	**p-OH-benzoic**	**Gallic**	**(n=3)**
P1	1.88 ± 0.10 B	0.88 ± 0.04 B (-17%)	0.64 ± 0.02 AB	0.85 ± 0.04 B	1.84 ± 0.08 A (-15%)	0.61 ± 0.08 A	0.29 ± 0.06 A	82 *(1.5)*
P2	2.23 ± 0.27 AB	0.85 ± 0.05 B (-20%)	0.60 ± 0.08 B	0.94 ± 0.04 AB	1.91 ± 0.10 A	0.64 ± 0.04 A	0.39 ± 0.03 A	88 *(1.1)*
P3	2.39 ± 0.22 AB	0.92 ± 0.02 AB	0.84 ± 0.12 AB	0.94 ± 0.06 AB	1.82 ± 0.06 A (-16%)	0.34 ± 0.03 B (-40%)	0.31 ± 0.08 A	89 *(1.9)*
P4	2.15 ± 0.06 AB	0.93 ± 0.02 AB	0.70 ± 0.06 AB	0.96 ± 0.05 A	1.93 ± 0.01 A	0.61 ± 0.06 A	0.34 ± 0.01 A	89 *(1.3)*
P5	2.49 ± 0.01 A	1.05 ± 0.03 A	0.61 ± 0.02 B	1.02 ± 0.01 A	1.98 ± 0.24 A	0.38 ± 0.04 B (-33%)	0.41 ± 0.01 A	94 *(1.0)*
P6	2.40 ± 0.09 AB	0.63 ± 0.13 C (-40%)	0.74 ± 0.06 AB	0.99 ± 0.03 A	2.10 ± 0.20 A	0.35 ± 0.01 B (-38%)	0.32 ± 0.01 A	88 *(1.9)*
P7	2.00 ± 0.40 B	0.71 ± 0.06 C (-33%)	0.84 ± 0.05 A	1.01 ± 0.07 A	1.88 ± 0.06 A	0.71 ± 0.04 A (+21%)	0.36 ± 0.02 A	88 *(1.7)*

The quantification of the main identified compounds in the biscuits, is reported in **Table 6**; in the last column, the recovery of polyphenols (expressed as percentage) after baking, is also reported.

The concentration of CH polyphenols changed in the biscuits differently after baking, depending on the powder used. The biscuit B1, prepared with the extract spray-dried without SAs, suffered the highest loss of total polyphenols, although it showed the highest amount of epicatechin. On the contrary, the biscuit B2, baked with the powder encapsulated with 80% MD, showed the highest amount of polyphenols, but the lowest epicatechin content. In general, baking affected the degradation of epicatechin and procyanidin B2, as occurs also during the roasting of cocoa beans, because these molecules are more susceptible to heat treatments (Kothe, Zimmermann, & Galensa, 2013). On the other hand, the catechin content increased in some of the biscuits while remaining constant in the others, probably due to the epimerization of epicatechin to catechin, that usually occurs during the roasting of cocoa (Kofink, Papagiannopoulos, & Galensa, 2007). All other compounds did not display an expected trend, except for the increase of gallic acid in all biscuits, that was previously observed also during the roasting of hazelnuts (Locatelli, Coisson, Travaglia, Bordiga, & Arlorio, 2015), and could be due to the degradation of polymerized polyphenols (hydrolyzable tannins), or to the hydrolysis of other glycosylated flavonoids (Schmitzer, Slatnar, Veberic, Stampar, & Solar, 2011). In general, the recovery of these compounds in the biscuits ranged from 62% of sample B4 to 104% of B2. Therefore, P2 was the most performing powder to formulate enriched biscuits

Table 6 Characterization of main CH polyphenols recovered following baking (B1-B7). Data are expressed as mg/g DE. Different letters indicate significant differences within each column ($p < 0.05$). Recovery values are calculated by comparing the sum of each compounds after baking to the value of the corresponding powder.

	Monomeric flavanols		**Procyanidins**		**Phenolic acids**			**Recovery (%)** ***(RSD %)***
	Epicatechin	**Catechin**	**B1**	**B2**	**Protocatechuic**	**p-OH-benzoic**	**Gallic**	**(n=3)**
B1	1.42 ± 0.13 A	0.99 ± 0.09 AB	0.60 ± 0.01 B	0.30 ± 0.02 B	0.41 ± 0.01 C	0.58 ± 0.02 B	0.38 ± 0.03 C	67 *(8.6)*
B2	0.10 ± 0.01 D	1.31 ± 0.07 A	0.95 ± 0.04 A	0.21 ± 0.01 C	0.71 ± 0.06 B	0.81 ± 0.03 A	3.79 ± 0.04 A	104 *(9.3)*
B3	0.92 ± 0.04 B	0.92 ± 0.08 B	0.48 ± 0.05 B	0.22 ± 0.01 C	0.50 ± 0.05 C	0.78 ± 0.03 A	3.09 ± 0.35 A	90 *(8.9)*
B4	0.50 ± 0.05 C	1.06 ± 0.12 AB	0.41 ± 0.04 B	0.38 ± 0.02 A	0.69 ± 0.01 B	0.71 ± 0.02 AB	1.01 ± 0.11 BC	62 *(5.8)*
B5	1.20 ± 0.09 A	0.97 ± 0.04 B	0.91 ± 0.08 A	0.33 ± 0.01 AB	0.79 ± 0.01 AB	0.74 ± 0.02 A	1.31 ± 0.03 B	78 *(5.1)*
B6	1.16 ± 0.04 AB	0.95 ± 0.09 B	0.89 ± 0.09 A	0.34 ± 0.03 AB	0.90 ± 0.00 A	0.67 ± 0.03 AB	1.29 ± 0.11 B	82 *(5.0)*
B7	0.48 ± 0.04 C	0.88 ± 0.05 B	0.90 ± 0.09 A	0.19 ± 0.01 C	0.69 ± 0.07 B	0.73 ± 0.08 A	3.45 ± 0.29 A	97 *(7.9)*

3.3 Morphological analysis of "P2" microencapsulated powder

The sample P2, the best performing powder in terms of oxidative protection of cocoa polyphenols during storage and baking, was finally characterized for the size, shape and surface of the microspheres. The microspheres showed a circularity of 0.9 and an elongation of 0.1, confirming a spherical shape and a diameter of 6.4 ± 0.6 μm.

SEM analysis showed a dented and collapsed surface (**Fig. 4**) attributable to the shrinkage of the particles during the drying process. A similar morphology was also observed by Robert et al. (2010) for pomegranate polyphenols spray-dried with MD. The microspheres resulted often aggregated in agglomerates, probably depending on the hygroscopic nature of the matrix. Moreover, the absence of breakages on the surface indicates that the powder might be characterized by a good stability.

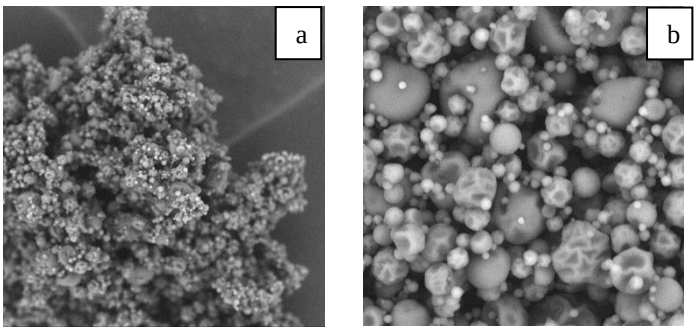


Figure 4 SEM images of the P2 microcapsules. Magnification: 2600x (a) and 8700x (b).

4 CONCLUSIONS

Cocoa hulls are a good source of antioxidant polyphenols, but these compounds are unstable to heat and air exposure. The preliminary results obtained in the present work confirm the usefulness of the microencapsulation process to limit the loss of polyphenols and antioxidant capacity. Particularly, the extract of cocoa hulls spray-dried with maltodextrins, in a coating to extract ratio of 80:20 (w/w), gives the best microspheres, in terms of protecting the polyphenols to the effect of storage (90 days) and baking (in a model biscuit). These results suggest the use of spray-dried extract of cocoa hulls as functional ingredients to enrich in polyphenols bakery products, and open to new interesting possibilities of application in food industry.

Future studies are needed to increase the polyphenolic content of these microencapsulated extracts and test their stability in other food matrices, such as leavened products, also evaluating rheological and organoleptic properties.

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Chapter 4

Manuscript in preparation

Spray-dried polyphenolic extract from Italian black rice (*Oryza sativa* L., var. Artemide) as functional ingredient for bakery products

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Abstract

Artemide rice is the Italian pigmented variety richest in polyphenols, especially anthocyanins. The aim of this work was to obtain anthocyanin-rich powders from Artemide cv, useful as ingredients in bakery functional foods.

A rice hydroalcoholic extract was powdered by spray drying with and without stabilizing agents, like maltodextrins (MD) and arabic gum (AG), and by freeze-drying, in order to obtain powdered ingredients, stable to storage and to baking.

The polyphenols spray-dried with MD and GA (50:50, w/w) resulted stable to the storage and partially protected during the baking in a model biscuit. The enriched biscuits showed a significantly higher content of polyphenols, antioxidant capacity and anthocyanins respect to the control biscuit. The polyphenolic extract obtained from Artemide black rice can be considered a valuable source of polyphenols to produce microencapsulated ingredients for nutraceutical applications.

Keywords

Anthocyanins, microencapsulation, antioxidant capacity, functional foods

INTRODUCTION

In the recent years, pigmented rice varieties have received significant attention for their beneficial effects on human health. Several studies report that the consumption of pigmented rice can promote the decrease of the oxidative stress, prevent the inflammation and reduce the risk of developing chronic diseases, like cardiovascular disease, type 2 diabetes and some forms of cancer (Dipti et al., 2012; Samyor, Das, & Deka, 2017).

There are several rice cultivars that contain pigments; most of them come from Asia, but their cultivation is also increasing in some Italian regions, like Piedmont and Lombardy.

The main molecules involved in color development are anthocyanins, generally localized in the external layer (bran). The color of the grain ranges from light red to dark purple/black, depending on the type and amount of anthocyanins. In addition to imparting color to plants, anthocyanins are responsible for an array of health-promoting benefits, as they can protect against a variety of oxidants through several mechanisms. Pigmented-rice is also characterized by other antioxidant compounds, such as flavones, proanthocyanidins and phenolic acids, which contribute to its healthy nutritional profile (Abdel-Aal, Young, & Rabalski, 2006; Dipti et al., 2012).

"Artemide" is an Italian black rice cultivar (*Oryza sativa* L.) obtained from the natural hybridization between the Venere rice (medium grain and black pericarp) and an indica ecotype (long and narrow grain, white pericarp). It is characterized by a higher content of polyphenols, particularly anthocyanins (cyanidin-3-glucoside as main compound), and a major antioxidant capacity, compared to other Italian pigmented varieties (Bordiga et al., 2014).

Anthocyanins are a class of flavonoids highly reactive and strongly subjected to degradation; their stability depends on environmental and chemical factors, such as pH, metal ions, exposure to light and UV, temperature, oxygen and

enzymatic activity (Cavalcanti, Santos, & Meireles, 2011). Consequently, due to their low stability during processing and storage, the direct use of these compounds in food formulation, especially in aqueous systems, is challenging (Mahdavi, Jafari, Assadpoor, & Dehnad, 2016).

Microencapsulation might be an efficient strategy to improve the stabilization of these compounds. Encapsulation is a technique to entrap and protect an active agent within another substance, called wall material or coating, working as carrier and stabilizing agent. It is a particularly fitting solution when it comes to antioxidant compounds, to protect the bioactive agents from adverse environmental conditions, thus limiting the oxidation and the loss of bioactivity and increasing the shelf life of the product (Kuang, Oliveira, & Crean, 2010). The most common microencapsulation technique used in food industry is spray-drying, which produces dry particles with diameters between few micrometers and few millimeters (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). In food sector, different types of carrier agent are used for the microencapsulation, such as proteins, carbohydrates and lipids. The selection of these substances depends on the purpose of the microencapsulation and on the physical-chemical characteristics of the encapsulated and encapsulating substances (Nedović, Kalušević, Manojlović, Petrović, & Bugarski, 2013). Anthocyanins are hydrophilic compounds compatible with water-based gel formulations, such as gums and maltodextrins. Maltodextrins (MD) have high water solubility and low viscosity, and provide a good oxidative stability to the encapsulated bioactive compound. These properties make them the most commonly used carrier materials in food area. Gum Arabic (GA) is another well-known material used since many years for microencapsulation, and still considered a preferred choice, because of its capacity to form stable emulsion. The selection of a polymeric mixture often leads to an improved encapsulation, particularly when a single wall

matrix does not possess all the required features to yield a proper encapsulation (Fang & Bhandari, 2011).

The main aim of the present work was to obtain anthocyanin-rich polyphenolic extracts from Artemide black rice, useful to produce functional foods. For this purpose, we prepared powdered extracts with and without stabilizing agents (MD, GA and MD/GA) by spray drying, to obtain stable extracts for bakery foods. Firstly, we studied the stability of the polyphenolic components, particularly the anthocyanin fraction, to the drying process and storage; then, the powdered extracts were tested as functional ingredients in a bakery model food (biscuit).

MATERIAL AND METHODS

2.1 Materials

Artemide rice was provided by the 'Azienda Agricola Luigi e Carlo Guidobono Cavalchini, tenuta La Mondina', in Casalbeltrame, Novara (Italy). Ethanol, methanol, Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical, sodium acetate, potassium chloride, ferric sulfate [$Fe_2(SO_4)_3 \cdot 5H_2O$], hydrochloric acid, formic acid, acetonitrile, GA, and all analytical standards were purchased from Sigma Aldrich (St. Louis, MO, USA), while MD (dextrose equivalent 16-20%) were obtained from A.C.E.F. (Fiorenzuola d'Arda, Italy).

2.2 Extraction of the polyphenolic fraction from rice

Polyphenols were extracted from Artemide rice, previously milled by a Tecator Cyclotec 1093 laboratory mill (International PBI, Milan, Italy) equipped with a 500-μm sieve, using water/ethanol 50:50 (v/v) in a 1:5 (w/v) ratio of sample to solvent. The extraction was performed under magnetic stirring for 15 minutes and in an ultrasonic bath (Branson 1510, Branson Ultrasonics, Danbury, CT) for another 15 minutes. Finally, the hydroalcoholic extract was filtered through a Whatman No. 1 filter paper. The dry matter of the extract (dry extract, DE) was

determined after drying at 105 °C in a ventilated oven, until constant weight was reached. Part of this extract was stored for 30 days at -20 °C (t_{30}), while the rest was powdered.

2.3 Preparation of powdered extracts

Firstly, the hydroalcoholic extract was concentrated by removing ethanol and part of the water using a rotary evaporator (Büchi® R-210, Flawil, Switzerland) at temperature lower than 40 °C, in order to facilitate the following drying process; then, the DE was determined, as previously described, in order to calculate the right amount of stabilizing agents to add. Successively, the concentrated extract was powdered as such (by both freeze-drying and spray-drying), or microencapsulated by spray-drying using MD, GA and a mixture of MD and GA (50/50, w/w) as stabilizing agents. Part of each powder was stored at -20 °C.

2.3.1 Spray-drying

Concentrated extracts were dried by a Mini Spray Dryer B-290 *(*Büchi®, Flawil, Switzerland) equipped with a 0.7 mm diameter nozzle, adding or not stabilizing agents, such as MD, GA and MD/GA (50/50, w/w). The ratio of the stabilizing agents respect to the DE was calculated at 80/20 (w/w) by preliminary findings, and kept constant in all the formulations. The non-encapsulated spray-dried powder (P-SD) and the microencapsulated extracts (P-MD, P-GA and P-MD/GA) were obtained using the same operative conditions. Spray drying was performed at 150 °C inlet temperature; feed flow and airflow were 7 mL/min and 40 m^3/h, respectively. In order to verify the microparticle integrity, spray-dried extracts were also analyzed morphologically and characterized by field emission scanning electron microscopy (FESEM) (Fig. S1).

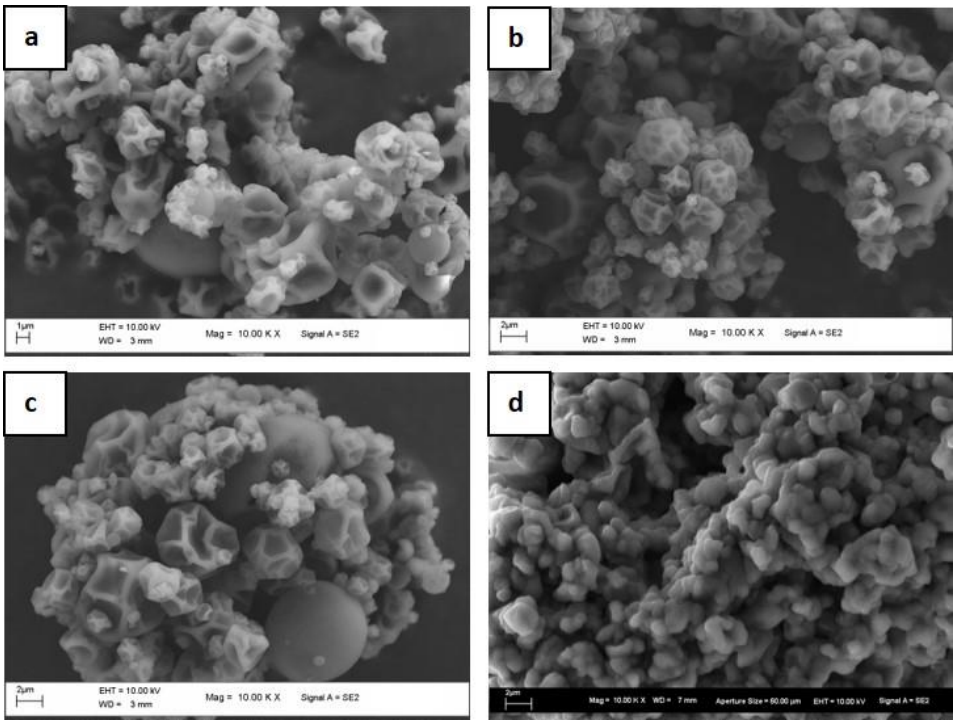

Figure S1 FESEM images of the powdered extracts: P-GA (a), P-MD (b), P-MD/GA (c), P-SD(d). The magnification was 5000x.

2.3.2 Freeze-drying

The concentrated extract was freeze-dried using a Heto PowerDry DW8 freeze dryer (Thermo Fisher Scientific, Massachusetts, USA), without adding the stabilizing agents. Freezing was carried out at -40 °C for 6-9 hours, followed by the primary drying at -35 °C for 20 hours with a chamber pressure of 1.11 mbar and a secondary drying at 20 °C for 5 h. The obtained powdered extract was labelled as P-FD.

2.4 Extraction of polyphenolic compounds from the powdered extracts

Polyphenols were extracted from the microencapsulated powders using the method described by Zhang, Mou, and Du (2007) with some modifications. Briefly, 30 mg of powder were extracted in an ultrasonic bath (Branson 1510, Branson Ultrasonic) using 1.5 mL of distilled water for 10 min, then the sample

was centrifuged at 2800 *x g* for 5 min at 4 °C and the supernatant was removed. Afterward, 1.5 mL of a solution methanol/water (80:20) were added to the residual pellet, ultrasonicated for 20 min and subsequently centrifuged. The two supernatants were combined and analyzed as described below; each powder was extracted in triplicate. Non-encapsulated powdered extracts (P-SD and P-FD) were solubilized similarly.

2.5 Formulation of a bakery model food (biscuit)

The three microencapsulated (P-MD, P-GA e P-MD/GA) and the non-encapsulated P-SD powders were used separately to formulate model biscuits (B-MD, B-GA, B-MD/GA and B-SD, respectively), following the AACC method (Gaines, 1986) with some modification. The selected recipe listed: wheat flour (55.2%), sugar (13.7%), shortening (29.5%) and an amount of powdered extract as to reach the 0.32% of DE in the crude biscuit. The powdered extract was replaced with flour in the recipe of the control biscuit (CB). The flour and the powdered extract were mixed with a pin mixer (1 min), sugar and shortening were mixed for 1 min, combined to the flour and mixed for other 5 min. The dough was rolled to a thickness of 0.7 mm and a diameter of 4.5 cm. Baking was carried out in a ventilated oven at 180 °C for 25 min. An image of cooked biscuits is reported in the supplementary materials (Fig. S2).

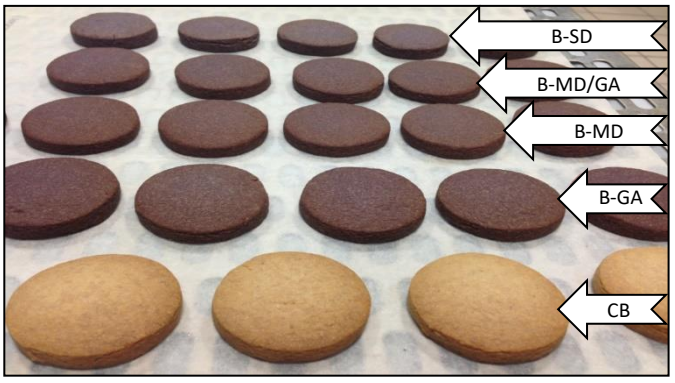

Figure S2 Image of the cooked biscuits

2.6 Extraction of polyphenolic compounds from the biscuits

The biscuits were firstly ground and defatted. Lipid fraction was extracted for 6 h using a semiautomatic Soxhlet extraction system B-811 (Büchi®, Flawil, Switzerland), employing dichloromethane as solvent. Two grams of each defatted biscuit were extracted twice: firstly, 6 mL of ethanol/water (50:50) were added, suspensions were vortexed for 1 min and centrifuged at 15000 *x g* for 2 min at 4 °C and the aqueous supernatants were recovered. Pellets were dissolved in 6 mL of methanol for 10 min in an ultrasound bath (Branson 1510, Branson Ultrasonic) and centrifuged again. The two supernatants were merged and analyzed. Each biscuit was extracted in triplicate.

2.7 Analytical methods

Total monomeric anthocyanins (TMA), total polyphenols (TP), condensed tannins (CT) content and antioxidant capacity (AOC) were determined using spectrophotometric methods. Main individual polyphenols were characterized by RP/HPLC-DAD on polyphenolic extract and powdered extracts at t_0, and on model biscuits.

2.7.1 Total polyphenols

The TP were determined using according to the modified version of the Folin–Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventos, 1999). on Artemide polyphenolic extract immediately after preparation (t_0) and after storage at -20 °C (t_{30}), on powdered extracts at t_0 and at t_{30}, and on model biscuits. One hundred microliters of Folin–Ciocalteu reagent and 350 µL of aqueous Na_2CO_3 (5% w/v) were added to an opportune volume of the sample, and the solutions were diluted to a final volume of 2.9 mL with distilled water. After one hour the absorbance was read at 760 nm, using an Evolution 60S spectrophotometer

(Thermo Fisher Scientific, Massachusetts, USA). Results were expressed as catechin equivalents (CE) through a calibration curve.

2.7.2 Total monomeric anthocyanins

The content of TMA was determined by the pH differential method, described in detail by Giusti and Wrolstad (2001) on Artemide polyphenolic extract and powdered extracts at t_0 and at t_{30}, and on model biscuits. Different samples were opportunely diluted with potassium chloride buffer (0.025 M), pH 1.0, until the absorbance of the sample at 520 nm was within the linear range of the spectrophotometer (*sample should not exceed 20% of the total volume).* The same dilution factor (DF) was applied to the dilution with sodium acetate buffer (0.4 M), pH 4.5. The two solutions were let to equilibrate for 5 and 15 min respectively, and the absorbance of both solutions was measured at 520 and 700 nm. TMA were expressed as cyanidin-3-glucoside (CY-3-Glu) equivalents and the concentration calculated through the formula:

TMA (µg/mL) = (*A* × 449.2 × DF × 1000)/(ε × 1)

where:

A = ($A_{520nm} - A_{700nm}$) at pH 1.0 – ($A_{520nm} - A_{700nm}$) at pH 4.5; 449.2 = molecular weight of CY-3-Glu; 1000 = conversion factor from g to mg; ε (molar extinction coefficient of CY-3-Glu) = 26900 L mol^{-1} cm^{-1}.

2.7.3 Condensed tannins

CT were determinated spectrophotometrically by the butanol-HCl method (Porter, Hrstich, & Chan, 1985), with minor modifications, on Artemide polyphenolic extract and powdered extracts at t_0 and at t_{30}. Reaction mixtures consisted of 1.0 mL of sample opportunely diluted in water and 3.0 mL of n-Butanol/HCl (50:50, v/v) 0.37-mM $Fe_2(SO_4)_3 \cdot 5H_2O$ solution. The mixtures were heated in a water bath at 95 °C for 30 min in covered test tubes and then cooled

in ice water; corresponding unheated reaction mixtures were used as blank controls. Absorbance was recorded at 550 nm, and CT content was determined as reported by Travaglia, Bordiga, Locatelli, Coisson, and Arlorio (2011) using the formula:

$CT\ (mg/mL) = (Abs_{sample} - Abs_{blank}) \times 0.1736 \times DF$

where DF is the dilution factor and 0.1736 is the conversion factor calculated from a non-commercial procyanidin solution.

2.7.4 Antioxidant capacity

The AOC was measured using the DPPH• radical scavenging assay, according to the method described by M. Locatelli et al. (2009), on Artemide polyphenolic extract and powdered extracts at t_0 and at t_{30}, and on model biscuits. Briefly, 700 µL of diluted sample or methanol (control) were added to the same volume of a 100 µM DPPH• methanolic solution. The solutions were vigorously shaken and left in the dark at room temperature. After 20 min of reaction, the absorbance was read at 515 nm and the DPPH• inhibition percentage was calculated. The AOC was expressed as Trolox equivalents (TE) by means of a calibration curve.

2.7.5 RP-HPLC/DAD analysis

The identification and quantification of polyphenols was carried out following the procedure described by Monica Locatelli, Travaglia, Coisson, Bordiga, and Arlorio (2016). A Shimadzu LC-20A Prominence chromatographic system, equipped with a diode array detector (DAD detector SPD-M20A), was used to analyze the main phenolic compounds. Separation was performed on a Synergi Max-RP column (4 µm, 80 Å, 250 x 4.6 mm; Phenomenex, Torrance, California, USA), protected by a guard column containing the same phase; the column temperature was set at 30 °C. The eluents were: (A) water/acetonitrile/formic acid (87:3:10) and (B) water/acetonitrile/formic acid (40:50:10). The gradient program

was set as follows: 0–20 min, 6–20% B; 20–35 min, 20–40% B; 35–40 min, 40–60% B; 40–45 min, 60–90% B; 45–50 min isocratic 90% B. Finally, the mobile phase was brought to 6% B in 0.5 min, and this was followed by 22.5 min of equilibration step. The mobile phase flow rate was 0.5 mL/min at 30 °C of temperature. Chromatograms were recorded at 280, 330 and 520 nm. Phenolic compounds were identified by comparing the retention times and UV/Vis spectra of their respective standards.

The quantification of the anthocyanins (CY-3-Glu, cyanidin-3-O-rutinoside, cyanidin-3,5-di-O-glucoside, delphinidin-3-O-glucoside, peonidin-3-O-glucoside) was performed at 520 nm. The quantification of *p*-coumaric acid and ferulic acid was performed at 330 nm, while syringic acid, protocatechuic acid, vanillic acid and sinapic acid were read at 280 nm. The flavonols (quercetin and myricetin) were quantified at 330 nm. Calibration curves of each compound, at six different concentration levels, were used for the quantification, except for cyanidin-3-O-rutinoside and cyanidin-3,5-di-O-glucoside, which were quantified as CY-3-Glu equivalents.

2.8 Statistical analysis

Results were reported as mean ± standard deviation of at least three independent experiments. The significance of differences was assessed by ANOVA, followed by Tukey's post hoc test ($p < 0.05$), using XLStat 365 (Addinsoft, Paris, France).

RESULTS AND DISCUSSION

3.1 Characterization of polyphenolic extracts

Artemide is one of the Italian pigmented rice variety richest in polyphenols, especially anthocyanins (Bordiga et al., 2014), and can be therefore considered an interesting source of antioxidant compounds. In the present work, we prepared a hydroalcoholic extract form Artemide rice and assayed different powdering methods (freeze versus spray drying, with or without stabilizing agents), with the aim of obtaining a stable anthocyanin-rich ingredient for the production of functional foods.

The hydroalcoholic extract and the corresponding powders were firstly analyzed at t_0 for their TP, TMA, CT content and AOC (**Table 1**, results are expressed on a DE basis). The hydroalcoholic extract showed a TMA concentration of 90.6 µg CY-3-Glu per gram of DE, corresponding to 2.4 g of CY-3-Glu per kg of rice, similar to that observed by Finocchiaro, Ferrari, and Gianinetti (2010) for Artemide rice, and comparble to that of blueberries (Siriwoharn, Wrolstad, Finn, & Pereira, 2004), fruits well-known for their high content of anthocyanins. Even the TP content and the AOC of hydroalcoholic extract (406.7 mg of CE and 555.6 mg of TE per gram of DE respectively, corresponding to 10,9 grams of CE and 59,9 mmol of TE per kilogram of rice respectively) were in accordance with previous literature data (Bordiga et al., 2014; Finocchiaro et al., 2010). Furthermore, the hydroalcoholic extract showed a CT content of 397.0 mg per gram of DE; this value is not directly comparable with others from the literature because of the different protocols employed.

The TP content of the powdered extracts, with and without stabilizing agents, did not show significant changes ($p>0.05$) after the drying processes, when compared to the hydroalcoholic extract measured at t_0. Contrarily, the AOC and the CT decreased significantly following microencapsulation. TMA decreased in

the microencapsulated extracts and in P-FD, but not in P-SD, showing a relative stability at the operative spray-drying conditions. The lower values registered in the microencapsulated extracts could be explained by a potential underestimation due to a non-exhaustive extraction, despite the use of ultrasounds and despite two consecutive runs of extraction. A similar result was achieved by Zhang et al. (2007), who obtained a productivity rate that ranged from 58% to 86% from the extraction of procyanidins microencapsulated with MD and GA.

Table 1 Total polyphenols, anthocyanins, condensed tannins and antioxidant capacity of hydroalcoholic and powdered extracts at t_0. Values are expressed as mg/g of DE. Different letters represent significant differences within each row ($p<0.05$).

	HYDRO ALCOHOLIC EXTRACT	POWDERED EXTRACTS				
		NON-ENCAPSULATED		*MICROENCAPSULATED*		
mg/g DE		P-SD	P-FD	P-GA	P-MD	P-MD/GA
Total polyphenols	407±32 ^{a}	399±28 ^{a}	415±11 ^{a}	340±35 ^{a}	331±38 ^{a}	351±19 ^{a}
Anthocyanins	90.6±6.2 ^{a}	80.5±0.8 ^{ab}	72.8±5.1 ^{bc}	73.0±2.7 ^{bc}	63.1±3.7 ^{c}	72.5±3.2 ^{bc}
Condensed tannins	397±33 ^{a}	398.±10 ^{a}	392±9 ^{a}	290±23 ^{b}	292±36 ^{b}	303±28 ^{b}
Antioxidant capacity	556±61 ^{a}	516±22 ^{ab}	515±10 ^{ab}	422±39 ^{b}	422±47 ^{b}	448±35 ^{b}

The phenolic characterization of the samples was completed by HPLC-DAD analysis, in order to identify and quantify the main anthocyanins, phenolic acids and flavonols (Table 2). In accordance with what reported by Bordiga et al. (2014), the most abundant anthocyanin recovered in all samples was the cyanidin-3-O-glucoside, and its concentration in the hydroalcoholic extract was 56.8 mg per gram of DE, representing the 86% of the anthocyanins identified in the extract. Contrarily, the cyanidin-3,5-di-O-glucoside was the least represented

anthocyanin, accounting for only 15.6 µg per gram of DE. The HPLC determinations confirmed a significant decrease of anthocyanins after drying. Comparing the different microencapsulated extracts, GA showed a greater protection of anthocyanins than MD. Nevertheless, the decreasing trend observed for each anthocyanin was similar in all extracts, with a concentration of cyanidin-3-O-rutinoside and peonidin-3-O-glucoside 16 and 12 times lower than the one of cyanidin-3-O-glucoside, respectively.

Protocatechuic acid was confirmed the most abundant phenolic acid in all the extracts (Sompong, Siebenhandl-Ehn, Linsberger-Martin, & Berghofer, 2011), ranging from 1.4 to 0.8 mg/g DE in P-GA and P-SD, respectively. Ferulic, vanillic, coumaric and sinapic acids were also identified, but they were 6, 13, 22 and 40-fold less abundant than the protocatechuic acid. In general, in our experiments, the drying processes carried out without stabilizing agents led to a significant decrease of phenol-acids when compared to the hydroalcoholic extract, while the presence of stabilizing agents (P-GA, P-MD and P-MD/GA) positively affected the stability of these molecules. A similar result was observed also for the myricetin and quercetin, which remained quite stable in the microencapsulated extract.

Table 2 Concentration of main phenolic compounds in the hydroalcoholic and powdered extracts at t_0. Values are expressed per gram of DE. Different letters represent significant differences within each row ($p<0.05$).

	HYDROALCOHOLIC EXTRACT	POWDERED EXTRACTS				
		NOT ENCAPSULATED		*MICROENCAPSULATED*		
		P-SD	P-FD	P-GA	P-MD	P-MD/GA
mg/g DE						
Cyanidin-3-O-glucoside	56.8±0.8 ^{a}	50.9±0.9 ^{b}	41.7±0.5 ^{e}	48.3±0.8 ^{c}	45.3±0.6 ^{d}	49.0±0.5 ^{c}
Cyanidin-3-O-rutinoside	3.43±0.01 ^{a}	2.99±0.06 ^{b}	2.57±0.01 ^{d}	2.95±0.05 ^{b}	2.77±0.05 ^{c}	2.96±0.05 ^{b}
Peonidin-3-O-glucoside	4.60±0.02 ^{a}	4.13±0.02 ^{b}	3.40±0.01 ^{d}	3.95±0.29 ^{bc}	3.67±0.23 ^{cd}	4.01±0.20 ^{bc}
Protocatechuic acid	1.36±0.01 ^{a}	0.84±0.01 ^{c}	0.97±0.01 ^{b}	1.40±0.07 ^{a}	1.32±0.07 ^{a}	1.34±0.08 ^{a}
µg/g DE						
Cyanidin-3,5-di-O-glucoside	44.1±1.9 ^{a}	40.9±2.2 ^{b}	57.8±1.2 ^{a}	30.2±1.7 ^{b}	15.6±0.9 ^{c}	33.8±4.1 ^{b}
Ferulic acid	234±13 ^{a}	174±2 ^{b}	165±5 ^{b}	234±25 ^{a}	212±15 ^{a}	219±5 ^{a}
Vanillic acid	105±4 ^{a}	50.0±4.9 ^{d}	65.4±4.8 ^{c}	102±3 ^{a}	87.9±6.7 ^{b}	96.1±5.3 ^{ab}
Coumaric acid	61.5±4.1 ^{b}	57.4±2.4 ^{b}	55.5±2.2 ^{b}	75.9±2.5 ^{a}	79.4±2.3 ^{a}	77.0±4.5 ^{a}
Sinapic acid	33.5±1.4 ^{a}	31.6±1.3 ^{a}	32.5±1.3 ^{a}	36.3±5.7 ^{a}	34.5±4.5 ^{a}	32.4±2.6 ^{a}
Quercetin	220±5 ^{b}	179±1 ^{cd}	162±6 ^{d}	267±8 ^{a}	248±11 ^{a}	199±11 ^{bc}
Myricetin	93.2±0.7 ^{a}	78.9±6.9 ^{bc}	74.3±5.4 ^{c}	92.0±2.9 ^{a}	85.9±1.7 ^{abc}	88.7±5.1 ^{ab}

3.2 Polyphenol stability after storage

The content of TP, TMA, CT and the AOC of the hydroalcoholic and powdered extracts were also determined following storage for 30 days (t_{30}) at -20 °C, in order to evaluate their stability over time. Results are reported in Table 3 and expressed on the DE basis; statistically significant changes respect to the t_0 values ($p<0.05$) are also indicated and reported (in brackets) as variation percentage.

The microencapsulated extracts were not affected by the period of storage, while significant decreases were registered both for hydroalcoholic and for non-encapsulated extracts, despite the low temperature (-20 °C). P-FD and P-SD appeared a little more stable than the corresponding hydroalcoholic extract, for which we registered significant loss in all the parameters, particularly TMA (-25%), followed by AOC (-18%), TP (-14%) and CT (-8%). These results confirm a low stability of the phenolic compounds in solution, as previously reported (Gradinaru, Biliaderis, Kallithraka, Kefalas, & Garcia-Viguera, 2003), and highlight the protective role of GA and MD in microencapsulated rice extracts during storage. Similar results have been previously obtained for black currant polyphenols, which, after microencapsulation with MD, resulted stable during storage at 8 °C and 25 °C for 3 months (Bakowska-Barczak & Kolodziejczyk, 2011).

Table 3 Total polyphenols, anthocyanins, condensed tannins content and antioxidant capacity of hydroalcoholic and powdered extracts after storage. Values are expressed as mg/g of DE. Different letters represent significant differences within each row ($p<0.05$). Significant variation percentages ($p<0.05$), compared to the results at t_0, are reported in brackets.

		POWDERED EXTRACTS				
	HYDRO ALCOHOLIC	*NON-ENCAPSULATED*		*MICROENCAPSULATED*		
mg/g DE	EXTRACT	P-SD	P-FD	P-GA	P-MD	P-MD/GA
Total polyphenols	348±26 ^{a} (-14%)	332±31 ^{a} (-17%)	339±14 ^{a} (-18%)	3330±28 ^{a}	308±26 ^{a}	340±32 ^{a}
Anthocyanins	68.3±9.3 ^{a} (-25%)	74.8±1.6 ^{a}	63.2±1.9 ^{a} (-13%)	70.8±4.9 ^{a}	62.8±3.1 ^{a}	70.9±4.2 ^{a}
Condensed tannins	365±13 ^{a} (-8%)	349±22 ^{a} (-12%)	368±34 ^{a}	308±20 ^{ab}	272±38 ^{b}	325±16 ^{ab}
Antioxidant capacity	454±44 ^{ab} (-18%)	505±5 ^{a}	489±3 ^{ab}	390±41 ^{b}	416±51 ^{ab}	433±61 ^{ab}

3.3 Formulation of polyphenol-rich biscuits

Many studies were made on the stability of anthocyanins to heating, usually by putting an anthocyanin containing solution in a water bath at 100 °C (Patras, Brunton, O'Donnell, & Tiwari, 2010). In this work and for the first time, the stability of anthocyanin-rich spray-dried polyphenolic extracts was studied in a baked model food (biscuits cooked in oven at 180 °C for 25 min).

The previously described spray-dried extracts (P-SD, P-GA, P-MD and P-MD/GA) were used as functional ingredients, obtaining the biscuits B-SD, B-GA, B-MD and B-MD/GA. P-FD was not considered for the biscuit preparation, because of its low content of anthocyanins. The content of TP, TMA and the AOC

were calculated by subtracting the contribution of the control biscuit (CB, prepared without rice extracts) and the results were finally expressed on the DE basis.

Spectrophotometric determinations showed a significant degradation of the phenolic component during baking (Table 4). Compared to the values determined on the corresponding powder (Table 1), TP decreased from 61% in B-MD to 80% in B-SD. Comparing the data of B-SD to those relative to biscuits prepared with microencapsulated extracts, it seems that stabilizing agents can slow down the degradation of polyphenols, even though the anthocyanins concentration is comparable in all biscuits. CT were not quantified in the biscuits because the assay was not suitable for this kind of matrix, since an unusual brown color was produced after heating the sample at 95 °C.

Table 4 Total polyphenols, anthocyanins and antioxidant capacity of powdered extracts after baking. Values are expressed as mg/g of DE. Different letters represent significant differences within each analysis (row) ($p<0.05$). Significant variation percentages ($p<0.05$), compared to the results of the powders at t_0, are reported in brackets.

mg/g DE	**B-SD**	**B-GA**	**B-MD**	**B-MD/GA**
Total polyphenols	81.2±6.8 ^{b} (-80%)	129.0±4.4 ^{a} (-62%)	128.0±4.5 ^{a} (-61%)	122.0±4.6 ^{a} (-65%)
Anthocyanins	33.2±0.3 ^{a} (-58%)	32.9±0.1 ^{a} (-55%)	33.9±1.55 ^{a} (-46%)	31.1±0.8 ^{a} (-57%)
Antioxidant capacity	102±6 ^{b} (-80%)	132±15 ^{a} (-69%)	117±12 ^{ab} (-72%)	122±12 ^{ab} (-73%)

The baking effect was also evaluated on the individual phenolic compounds; the results, determined per gram of DE, are shown in Table 5. The cyanidin-3-O-glucoside was confirmed as the most abundant polyphenol (6.7 mg/g DE), representing the 88% of all anthocyanins in all powdered extracts. With the exception of cyanidin-3,5-di-O-glucoside, all the identified anthocyanins were significantly affected by the thermal treatment, showing a decrease of about 90% compared to corresponding powders at t_0. The higher stability of cyanidin-3,5-di-O-glucoside to high temperatures could be related to its double glycosylation, as previously suggested by different Authors (Hiemori, Koh, & Mitchell, 2009; Zaupa, Calani, Del Rio, Brighenti, & Pellegrini, 2015).

Considering phenolic acids, the concentration of protocatechuic acid significantly increased in all the samples considered, especially in the B-SD (+118%). Aside from its known stability to heating (Zaupa et al., 2015), the increase of concentration of this phenolic acid is attributable to the partial degradation of cyanidin-3-O-glucoside in protocatechuic acid occurring during the thermal treatment, as suggested by Hiemori et al. (2009). Vanillic acid also increased after baking, a similar result was observed after heating a citrus peel extract at 120 °C for 90 min (Xu, Ye, Chen, & Liu, 2007). On the contrary, ferulic, sinapic and coumaric acids significantly decreased, and also this result was in line with what observed by Finocchiaro et al. (2007) after the cooking of white and red rice. Even the flavonols, myricetin and quercetin showed a significant loss of about 31%, a drop typically observed after cooking the rice (Zaupa et al., 2015).

Table 5. Quantification of the main phenolic compounds of powdered extracts after baking. Values are expressed per gram of DE. Different letters represent significant differences within each row ($p<0.05$). Significant variation percentages ($p<0.05$), compared to the results of the powders at t_0, are reported in brackets.

mg/g DE	B-SD	B-GA	B-MD	B-MD/GA
Cyanidin-3-O-glucoside	8.40±0.14 [a] (-84%)	5.78±0.13 [c] (-88%)	5.87±0.11 [c] (-87%)	6.77±0.17 [b] (-86%)
Cyanidin-3-O-rutinoside	0.45±0.03 [a] (-85%)	0.28±0.01 [c] (-91%)	0.29±0.01 [c] (-90%)	0.34±0.00 [b] (-88%)
Peonidin-3-O-glucoside	0.67±0.01 [a] (-83%)	0.43±0.43 [d] (-89%)	0.47±0.02 [c] (-87%)	0.52±0.51 [b] (-87%)
Protocatechuic acid	1.83±0.04 [c] (+118%)	2.28±0.05 [b] (+62%)	2.39±0.05 [ab] (+81%)	2.51±0.01 [a] (+87%)
μg/g DE				
Cyanidin-3,5-di-O-glucoside	43.5±2.0 [a]	34.7±0.9 [b]	17.7±0.9 [c]	39.6±2.0 [a]
Ferulic acid	183±9 [a]	145±7 [b] (-38%)	150±5 [b] (-29%)	151±8 [b] (-31%)
Vanillic acid	143±6 [c] (+185%)	197±5 [a] (+98%)	177±1 [b] (+101%)	174±4 [b] (+81%)
Coumaric acid	61.7±4.5 [b]	64.0±3.5 [b] (-16%)	79.3±2.0 [a]	64.8±4.1 [b] (-16%)
Sinapic acid	26.8±0.5 [a] (-15%)	20.9±3.3 [a] (-42%)	23.0±2.6 [a] (-33%)	22.9±2.8 [a] (-29%)
Quercetin	176±2 [b]	203±2 [a] (-23%)	169±11 [b] (-32%)	184±5 [b]
Myricetin	59.2±6.0 [a] (-25%)	53.0±0.7 [a] (-42%)	52.4±5.6 [a] (-39%)	64.6±5.1 [a] (-27%)

Finally, concentration of polyphenolic compounds in the biscuits prepared with the powdered rice extracts were compared to those of control biscuit CB, in order to evaluate the enrichment in the polyphenol content; to this purpose, data were expressed on gram of biscuit (Table 6).

The spectrophotometric analysis showed a greater TP content, of about 75%, in the biscuits with microencapsulated polyphenols respect to the control. Biscuit prepared with P-SD, without stabilizing agents, showed instead an increase in TP of only 57%, about 20% lower than that of the microencapsulated powders, confirming the protective role of the stabilizing agents during baking (Idham, Muhamad, Setapar, & Sarmidi, 2012). Even the AOC displayed a similar trend, while the TMA were obviously present only in the enriched biscuits.

The HPLC results in Table 6 show that there was an enrichment in anthocyanins, flavonols and phenolic acid in all biscuits when compared to CB, although the baking caused the loss of polyphenolic compounds. Phenolic acids were the only compounds already present in the CB, because of the wheat flour used to prepare the biscuits (Arranz & Calixto, 2010).

Table 6. Characterization of the biscuits by spectrophotometric and HPLC analysis. Values are expressed as µg of phenolic compound on g of biscuit. Different letters represent significant differences within each row ($p<0.05$). The percentages of variation compared to CB are reported in brackets.

µg/g biscuit	**CB**	**B-SD**	**B-GA**	**B-MD**	**B-MD/GA**
Polyphenols	572±16 ^{d}	896±26 ^{c} (+57%)	1021±16 ^{a} (+78%)	1000±9 ^{a} (+75%)	975±13 ^{b} (+70%)
Anthocyanins	-	128±1 ^{a}	119±1 ^{ab}	122±4 ^{ab}	114±5 ^{b}
Antioxidant capacity	283±16 ^{c}	483±5 ^{b} (+71%)	599±18 ^{a} (+83%)	482±13 ^{b} (+70%)	498±14 ^{ab} (+76%)
Cyanidin-3-O-glucoside	-	32.2±0.6 ^{a}	20.9±0.5 ^{c}	21.0±0.4 ^{c}	24.4±0.6 ^{b}
Cyanidin-3-O-rutinoside	-	1.74±0.01 ^{a}	1.00±0.04 ^{c}	1.04±0.03 ^{c}	1.24±0.01 ^{b}
Peonidin-3-O-glucoside	-	2.58±0.05 ^{a}	1.56±0.05 ^{d}	1.68±0.06 ^{c}	1.88±0.03 ^{b}
Cyanidin-3,5-di-O-glucoside	-	0.26±0.02 ^{a}	0.21±0.02 ^{b}	0.21±0.01 ^{bc}	0.17±0.01 ^{c}
Protocatechuic acid	1.7±0.1 ^{d}	8.72±0.19 ^{c} (+404%)	9.94±0.18 ^{b} (+470%)	10.2±0.2 ^{ab} (+484%)	10.7±0.4 ^{a} (+512%)
Ferulic acid	1.48±0.10 ^{c}	2.22±0.10 ^{a} (+50%)	1.96±0.02 ^{b} (+33%)	1.95±0.004 ^{b} (+32%)	2.00±0.02 ^{b} (+35%)
Vanillic acid	0.28±0.02 ^{d}	0.84±0.04 ^{c} (+201%)	0.99±0.03 ^{a} (+258%)	0.90±0.01 ^{b} (+224%)	0.89±0.02 ^{bc} (+222%)
Coumaric acid	0.13±0.01 ^{c}	0.36±0.03 ^{b} (+180%)	0.36±0.01 ^{b} (+177%)	0.41±0.01 ^{a} (+215%)	0.36±0.01 ^{b} (+175%)
Sinapic acid	0.17±0.02 ^{c}	0.27±0.01 ^{a} (+56%)	0.24±0.01 ^{ab} (+38%)	0.25±0.01 ^{ab} (+47%)	0.24±0.01 ^{b} (+38%)
Quercetin	-	0.68±0.01 ^{b}	0.73±0.01 ^{a}	0.60±0.04 ^{c}	0.66±0.02 ^{b}
Myricetin	-	0.23±0.02 ^{ab}	0.19±0.00 ^{ab}	0.19±0.02 ^{b}	0.23±0.02 ^{a}

CONCLUSIONS

Concluding, the polyphenolic components of Artemide black rice can be spray dried to obtain powders useful as ingredients for functional foods. The microencapsulation of Artemide rice extract, with MD and GA (50:50, w:w) as stabilizing agents, protect and consequently stabilize the polyphenols during the storage at -20 °C for at least 30 days. Model biscuits enriched with powdered rice extracts show an improved polyphenolic profile, despite a partial degradation occurring during baking. In future, it will be necessary to test more stabilizing agents to obtain more performing functionalized ingredients to be employed in the bakery sector.

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Chapter 5

Manuscript in preparation

Evaluation of *in vitro* gastro-intestinal digestion and colonic biotransformation on the degradation of curcuminoids in different formulations and foods

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1 Introduction

Turmeric (*Curcuma longa* L.) is a rhizome extensively used as spice, food coloring and additive (Kocaadam & Sanlier, 2017). The main colored and bioactive compounds in turmeric are the curcuminoids: the curcumin [7-bis(4-hydroxy-3-methoxyphenil)-1,6-heptadiene-3,5-dione)], the demethoxycurcumin and the bisdemethoxycurcumin. They belong to the group of diarylheptanoids (or diphenylheptanoids) having an aryl-C7-aryl skeleton (Li et al., 2011).

Curcumin is considered the main active and abundant ingredient in turmeric; it has shown antitumor, antioxidant, antimicrobial and anti-inflammatory properties, both in *in vitro* and in *in vivo* studies (Kocaadam et al., 2017).

Curcumin is produced industrially from turmeric oleoresin, which is a brownish-orange viscous oily product extracted from the rhizome with a non-aqueous solvent (it may be ethanol, methanol, acetone, isopropanol, dichloromethane or hexane), followed by the removal of the solvent by evaporation (Jayaprakasha, Negi, Anandharamakrishnan, & Sakariah, 2001). Turmeric oleoresin is not water-soluble, it contains resinous material, volatile compounds, essential oils (Hastak et al., 1997) and about 30-45% (w/w) of curcuminoids (Kshirsagar, Yenge, Sarkar, & Singhal, 2009), which concentration depends from variety and cultivation conditions (Li et al., 2011).

Downsides of curcumin are represented by its low stability when exposed to light, high temperature, metallic ions, enzymes and oxygen (Y. Wang, Lu, Lv, & Bie, 2009), and by its low bioavailability, related to low bioaccessibility, high rate of metabolism, rapid elimination and clearance from the body (Lee et al., 2013). The low bioaccessibility, in turn, depends on low water-solubility (18 ng/mL) (Kaminaga et al., 2003) and susceptibility to degradation under alkaline conditions. Particularly, at pH above 7 and within thirty minutes, it degrades to trans-6-(40-hydroxy-30-methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic acid, feruloylmethane, and vanillin (Y. J. Wang et al., 1997). Moreover, under acidic

conditions, the degradation of curcumin is slower, with less than 20% of total curcumin decomposed in 1 hour (Y. J. Wang et al., 1997).

Therefore, when curcumin is orally ingested, only a small portion is absorbed within the intestine, metabolized and then excreted through urine (Hoehle, Pfeiffer, Solyom, & Metzler, 2006). The major portion of curcumin arrives to the colon where it is fermented by colonic bacteria, and more than 75% excreted through faeces (Lee et al., 2013).

Many strategies have been developed and applied to overcome the limitations of low stability and bioavailability of curcumin, including liposomes, nanoparticles and polymeric micelles, micro-emulsions and phospholipid-complexes (Liu et al., 2016). The latter is applied in Meriva®, a patented ingredient used in food supplements and drugs. Moreover, also the co-administering of curcumin with adjuvants like piperine and quercetin (Anand, Kunnumakkara, Newman, & Aggarwal, 2007), or the combination of the three curcuminoids with volatile oils present in oleoresin, led to an increase of curcumin absorption in humans (Jager et al., 2014). In a previous study (Vitaglione et al., 2012), the greatest *in vivo* bioavailability of curcuminoids, encapsulated by a double coating of cellulose derivate and vegetable oil, from enriched bread, was demonstrated. Furthermore, it is known that also the food matrix and the cooking method may affect the bioaccessibility and bioavailability of curcuminoids (Zou et al., 2015). Although many formulations have been developed to increase the bioavailability of curcuminoids, they are often produced for pharmaceutical and food supplement industry, using excipients or expesive technologies unsuitable for food industry.

In this work, we wanted study the *in vitro* bioaccessibility, using a standardised protocol recently, and the degradation of curcuminoids along the gastro-intestinal tract, in a new microencapsulated food ingredient (GA/MD) comparing it to two commercial ingredients, turmeric (low curcumin bioavailability) and Meriva®

(high curcumin bioavailability). The microencapsulated ingredient GA/MD was obtained by spray drying the turmeric oleoresin with gum arabic and maltodextrins. Moreover, to study the matrix effect, the degradation of curcuminoids was evaluated in two model foods, yogurt and rice, enriched with the food ingredients. To this purpose, *in vitro* gastro-intestinal digestion and microbial biotransformation in the three tracts of colon (ascending, transverse and descending) were simulated.

2 MATERIAL AND METHODS

2.1 Materials

The turmeric oleoresin was provided by Fiorio colori SpA (Milan, Italy), the turmeric powder by MB Med srl.

Meriva® was provided by Indena SpA (Milan, Italy). It is obtained by mixing curcumin, soy lecithin and microcrystalline cellulose in a 1:2:2 weight ratio, through the Phytosome® strategy, to improve the curcumin bioavailability. The plain yogurt and the "Carnaroli" rice were purchased at the supermarket. Ethanol, formic acid, acetonitrile, GA, and all analytical standards were purchased from Sigma Aldrich (St. Louis, MO, USA), while MD (dextrose equivalent 16-20%) was obtained from A.C.E.F. (Fiorenzuola d'Arda, Italy). The enzymes pepsin (3600U/mg) from porcine gastric mucosa, pancreatin (8 × USP) from porcine pancreas and the porcine bile extract were purchased from Sigma-Aldrich (Milan, Italy).

2.2 Microencapsulation of oleoresin by spray drying

To obtain the microencapsulated ingredient (GA/MD), turmeric oleoresin was microencapsulated with *food grade* wall materials in a 95:5 (w/w) ratio of GA and MD mixture and oleoresin. The GA and MD mixture, in a 80:20 (w/w) ratio,

was dispersed in water at 60 °C, with magnetic stirring until complete hydration, to a final concentration of 20% (w/v), and then kept in fridge overnight.

Turmeric oleoresin was solubilized in ethanol at a concentration of 2% (w/v), keep in constant agitation for 1 hour at room temperature in the dark. Once solubilized, it was added, dropwise, to the dispersion of wall materials, in a constant stirring at 1000 *rpm* for 10 min, then mixed using a rotor-stator homogenizer Ultra-Turrax® T25 Basic (Ika-Werke GmbH, Staufen, Germany) at 13500 *rpm* for 2 min and finally sonicated for 10 min in an ultrasonic bath to obtain a fine emulsion (Sari et al., 2015).

The spray-drying of the emulsion was obtained using the *Mini Spray Dryer B-290 (*Büchi®, Switzerland) and was performed at inlet and outlet temperature of 130 °C and 80 °C, with a feed flow of 7 mL/min and airflow of 40 m^3/h.

2.3 Formulation of foods enriched with ingredients

The ingredients were added to two model foods, plain yogurt and rice, at a curcuminoid concentration of 0.05% (w/w).

The plain yogurt was enriched with only GA/MD (Y-GA/MD), because the other two ingredients (Meriva® and turmeric) did not dissolve in this medium. To obtain homogeneous samples, the yogurt was kept in magnetic stirring, and when the ingredient was dissolved, the sample was treated with the rotor-stator homogenizer at 13500 *rpm* for 2 min. An aliquot of plain yogurt was used as control. The yogurt was stored at 4 °C and analyzed within five days. The rice was enriched with GA/MD (R-GA/MD) and turmeric (R-turmeric) to compare a commercial food ingredient to the microencapsulated one. One hundred grams of rice were cooked in 600 mL of boiling water for 14 min. The initial volume of water was determined prior to the experiment to make sure all water was absorbed by the rice at the end of cooking. The cooked rice was divided in three parts: one was added with the ingredient GA/MD, one with turmeric and one represented

the control (white rice). These rice samples were then roughly grinded to obtain a granulometry similar to that following the mastication and stored in fridge at 4 °C for five days. Before the *in vitro* digestion, the samples were kept at 60 °C for 10 min to simulate the conditions after cooking.

2.4 *In vitro* digestion

To evaluate *in vitro* bioaccessibility of curcuminoids, the standard INFOGEST protocol (Minekus et al., 2014) was applied to ten grams of food (Y-GA-MD, R-GA/MD and R-turmeric), 500 mg of GA/MD, 250 mg of turmeric and 50 mg of Meriva®, corresponding to 5 mg of curcuminoids. Samples were digested in triplicate. At the end of each digestive step, one milliliter of digested sample was collected, and the same fractions (stomach and small intestine) of three separate digestions were combined and mixed; the analyses were performed on the combined sample.

2.4.1 Determination of in vitro bioaccessibility

An aliquot of the sample after pancreatic digestion was centrifuged at 2300 *x g* for 5 min at 4 °C, to separate the soluble (supernatant) and insoluble (pellet) fraction. The bioaccessibility of curcuminoids was calculated as follows (Ortega, Reguant, Romero, Macia, & Motilva, 2009):

= (Total curcuminoids of soluble fraction/Total curcuminoids in small intestine) x 100

2.5 Twin-SHIME®

The Simulator of Human Intestinal Microbial Ecosystem (SHIME®) was used to simulate the three tracts of colon: ascending, transverse and descending.

It was set up using two vessels for each tract (Twin-SHIME®) and all vessels were inoculated with the same faecal sample of a healthy human volunteer.

Inoculum was prepared from freshly voided faecal samples diluted 10-fold in 0.1 M phosphate pH = 7.0 with 1 g/L sodium thioglycolate and centrifuged briefly to remove particulates. SHIME® was fed three times a day with 200 mL of a solution composed of: 1 g/L arabinogalactan, 2 g/L pectin, 1 g/L xylan, 3 g/L potato starch, 0.4 g/L glucose, 3 g/L yeast extract, 1 g/L pepton, 4 g/L mucin and 0.5 g/L cysteine. The pH of the feed was set to 2 and the feed was stored at 4 °C before incubation in each vessel.

The system was used according to the condition reported in **Table 1**, in the hours spanning between the end of a flow and the beginning of the next one (static way). It was kept at 37 °C and in anaerobic condition by flushing it daily with N_2 for 15 minutes.

Table 1. Parameters of Twin-SHIME® vessels

	Volume (mL)	pH
Ascending	500	5.6–5.9
Transverse	800	6.1–6.4
Descending	600	6.6–6.9

The samples (ingredients and insoluble fractions of rice) were added in a right amount to have the same curcuminoid concentration (5 µg/mL) in each vessel. To allow the transfer in the vessels, samples were firstly hydrated with 2 mL of water. To analyze the microbial biotransformation over time, 5 mL of each vessel content were collected at 0, 10, 25, 50, 75, 100, 150, 225 and 300 min, for a total of 9 aliquots. Samples were stored at −20 °C until the analysis.

2.6 Extraction of curcuminoids

2.6.1 Extraction of curcuminoids from the ingredients

Fifty milligrams of microencapsulated ingredients were firstly hydrated with 400 μL of water by vortex, extracted with 1600 μL of ethanol, sonicated for 10 minutes and centrifuged at 20800 *x g* for 10 min at 4 °C. The supernatant was conveniently diluted and analyzed.

One milligram of turmeric was extracted with 1 mL of ethanol/water 80:20 (v:v), sonicated for 5 min and centrifuged at 20800 *x g* for 10 min. The pellet was further extracted with 1 mL of ethanol, sonicated for 5 min and centrifuged. The two supernatants were combined, diluted and analyzed.

One milligram of Meriva® was extracted with 1 mL of ethanol/water 80:20 (v:v), sonicated for 5 minutes e centrifuged at 20800 *x g* for 10 min. The supernatant was diluted and analyzed.

2.6.2 Extraction of curcuminoids from the yogurt

Five hundred milligrams of yogurt were extracted firstly with 1.5 mL ethanol, mixed by vortex for 1 min, sonicated for 5 min and then centrifuged at 20800 *x g* for 5 min. 1.5 mL of ethanol/water (80:20 v/v) were further added to the residual pellet, sonicated for 5 min and subsequently centrifuged. This step was performed twice and, finally, the three supernatants were combined, diluted and analyzed.

2.6.3 Extraction of curcuminoids from the cooked rice

One hundred milligrams of cooked rice were extracted firstly with 1.5 mL ethanol/water (80:20 v/v) in agitation for 5 min and then centrifuged at 2300 *x g* for 5 min. The supernatant was removed. Afterward, 3 mL of ethanol/water (80:20 v/v) were added to the residual pellet, sonicated for 5 min and subsequently centrifuged. The two supernatants were collected, centrifuged at 20800 *x g* for 5 min, diluted conveniently and analyzed.

2.6.4 Extraction of curcuminoids from digested samples

The curcuminoids were extracted from the samples at the end of each digestive step (stomach and small intestine). One hundred milligrams of sample were extracted with 2 mL of ethanol, mixed for 5 min and centrifuged at 20800 *x g* for 5 min. The supernatant was diluted and analyzed.

Five hundred microliters of soluble fraction after pancreatic digestion were treated with 0.5 mL of ethanol and mixed for 5 min. The sample was then centrifuged at 20800 *x g* for 5 min and then analyzed.

The insoluble fraction after pancreatic digestion was extracted by adding 3 mL of ethanol to 0.1 g of pellet, mixing for 5 min and centrifuging at 2300 *x g* for 5 min. After that, the pellet was extracted again with 2 mL of ethanol in an ultrasonic bath for 10 min, then centrifuged at 20800 *x g* for 5 min. The two supernatants were combined before the analysis.

2.6.5 Extraction of curcuminoids after colonic fermentation

The extraction of curcuminoids from 0.5 mL of sample incubated in the Twin-SHIME system was preceded by a step of centrifugation at 20800 *x g* for 5 min at 4 °C. Subsequently, the supernatant was eliminated, and the pellet was extracted with 0.5 mL of ethanol, vortexed and sonicated for 10 min. The sample was then centrifuged at 20800 *x g* for 5 min at 4 °C and the supernatant analyzed.

2.7 HPLC analysis

The determination of curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) was performed by a Thermo Finnigan Surveyor HPLC System (Thermo Scientific, MA, USA) equipped with a photodiode array (PDA) detector. Separation was performed on a reversed-phase Xbridge Shield 18 column (100 mm x 2.1, 3.5 µm; Waters, Massachusetts, USA). The eluents were: (A) acidified acetonitrile (0.1% formic acid) and (B) acidified water (0.1% formic

acid). The gradient program was: 0-5 min, 95-65 % B; 5-30 min, 65-50 % B; 30-31 min, 50-0 % B; 31-35 min, 0% B; 35-45 min, (0-95%); 45-51 min, 95% B. The flow rate of mobile phase was 0.5 mL/min and the column temperature of 30 °C. The injection volume was 5 µL. Chromatograms were recorded at 425 nm. All compounds were identified comparing retention time and UV/Vis spectra of each respective standard. Calibration curves of each compound, at six different concentration levels, were used for the quantification. Results were expressed as total curcuminoids, calculated as sum of the three curcuminoids.

2.8 SEM

The surface morphology of microencapsulated samples was carried out by a scanning electron microscopy (SEM) Phenom XL (Phenom-World, Eindhoven, Netherlands), equipped with two detector systems: the first one is a four-segment BackScatter Detector, fully integrated EDS system; the second one is a Secondary Electron Detector (SED). Samples of microspheres were placed directly on a SEM stubs "charge reduction".

2.9 Statistical analysis

Results were reported as mean ± standard deviation of at least three experiments. The significance of differences was determined by ANOVA, followed by Tukey's post hoc test ($p < 0.05$) using XLStat 365 (Addinsoft, Paris, France).

3 RESULTS AND DISCUSSION

3.1 HPLC characterization of ingredients and oleoresin

Turmeric oleoresin was microencapsulated with emulsifying and coating agents, GA and MD, to improve its water-solubility, stability and bioaccessibility.

Turmeric oleoresin, the microencapsulated ingredient (GA/MD) and the two commercial products (turmeric and Meriva®) were characterized for their curcuminoids by HPLC-DAD (**Tab. 2**). The microencapsulated ingredient and the oleoresin showed a similar composition: 65% of curcumin, 17% of demethoxycurcumin and 18% of bisdemethoxycurcumin. However, the concentration of total curcuminoids in the microencapsulated ingredient (1% w/w) was 24-fold less than that of oleoresin, and half the one of turmeric powder, with a slightly different composition (71%, 16% and 12% of curcumin, demethoxycurcumin and bisdemethoxycurcumin, respectively).

Table 2 Curcuminoids in oleoresin and ingredients. Values are expressed as mg of curcumin, demethoxycurcumin and bisdemethoxycurcumin per gram of sample. Percentages of each curcuminoid on total curcuminoids are reported in brackets. The concentration (w/w) of total curcuminoids is reported in the last column.

	Curcumin	**Demethoxy-curcumin**	**Bisdemethoxy-curcumin**	*% total curcuminoids*
OLEORESIN	160 ± 7 (66%)	41.2 ± 0.8 (17%)	39.6 ± 1.1 (17%)	*24*
GA/MD	6.20 ± 0.26 (63%)	1.75 ± 0.04 (18%)	1.89 ± 0.02 (19%)	*1*
Turmeric	14.4 ± 0.3 (71%)	3.26 ± 0.05 (16%)	2.49 ± 0.05 (12%)	*2*
Meriva®	168 ± 6 (82%)	27.3 ± 1.8 (13%)	8.69 ± 0.04 (5%)	*20*

3.2 Degradation of curcuminoids in the ingredients along the gastro-intestinal tract

GA/MD, turmeric and Meriva® underwent an *in vitro* digestion to analyze the degradation of curcuminoids, in different formulations, at physiological conditions (pH, temperature and type of enzymes). The results of total

curcuminoids for each formulation (undigested ingredient), after the gastric (pepsin) and the intestinal phase (pancreatin) are displayed in **Table 3**. All the ingredients registered a significant decrease in the amount of curcuminoids at the end of the two digestions, higher for GA/MD (-32%) and lower for turmeric and Meriva® (16-17%). The ingredient microencapsulated with GA/MD showed the highest loss already in the gastric phase (-20%), when compared to turmeric (-14%) and Meriva® (-9%). Similar results were observed also by Aniesrani Delfiya, Thangavel, Natarajan, Kasthuri, and Kailappan (2015), who highlighted that the curcuminoids microencapsulated with GA were released more quickly, in an acid medium, than those from oleoresin, because of the fast hydrolysis of curcumin-GA bond. Therefore, Meriva® was the formulation that showed the highest stability to the gastrointestinal conditions, due to the presence of phospholipids that protect the sensitive active agents from the degradation (Fricker et al., 2010).

Table 3 Effect of *in vitro* gastro-intestinal digestion on curcuminoid content of ingredients Values are expressed as mg of total curcuminoids per gram of digested ingredient. Percent variations of digested sample compared to the undigested are reported in brackets. Different letters represent significant differences within the same sample (column) ($p<0.05$).

	GA/MD	**Turmeric**	**Meriva®**
Undigested	10.3±0.8^{a}	20.1±0.4^{a}	204±8^{a}
Gastric phase	8.22±1.13^{b} (-20%)	17.3±1.3^{ab} (-14%)	186±15^{ab} (-9%)
Intestinal phase	7.05±0.77^{b} (-32%)	16.8±1.2^{b} (-16%)	169±7^{b} (-17%)

To determinate the bioaccessible fraction of total curcuminoids, the digested samples were separated in two fractions, soluble (bioaccessible) and insoluble

(non-bioaccessible). **Figure 1** shows the percentage of soluble and insoluble curcuminoids after the intestinal phase for GA/MD, Meriva® and turmeric. The solubility of GA/MD was 3%, 15-fold higher than the one of turmeric (0.2%) and similar to that of Meriva® (5%). These results highlighted the increased curcumin bioaccessibility in the ingredients GA/MD and Meriva®, where the curcuminoids are present in form of a micro-emulsion (Ahmed, Li, McClements, & Xiao, 2012; Zou et al., 2015), compared to the turmeric powder.

Besides, considering that the adequate daily intake (ADI) of curcumin of 0–3 mg per kg of body weight (EFSA 2014), a healthy individual of 70 kg should consume 4–10 g of turmeric powder daily. Taking in account that the bioaccessibility of the spray-dried ingredient resulted *in vitro* 15-fold higher than turmeric, although the spray-dried ingredient was only at 1% in curcuminoids, an amount of 0.5-1.3 g of this formulation alone could give the same amount of bioaccessible curcuminoids of 4-10 g of turmeric powder.

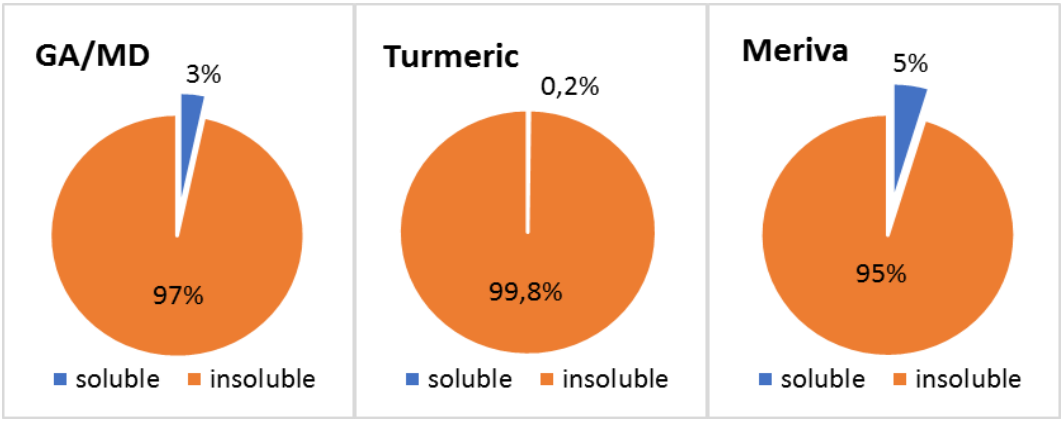

Figure 1. Bioaccessibility of curcuminoids after the *in vitro* gastro-intestinal digestion of the ingredients. Values are expressed as percentage of bioaccessible (soluble) and non-bioaccessible (insoluble) curcuminoids of small intestine fraction.

In order to study the single contribution of each tract of the colon to the degradation of curcuminoids, the three ingredients were incubated with a faecal inoculum in SHIME® vessels, so that the initial concentration of total curcuminoids for each sample was the same in each vessel (5 µg/mL). For the first time, the degradation of total curcuminoids in each colon tract is showed in **Figure 2**. After 5 hours of incubation, the loss of curcuminoids was about one third in the ascending colon (29% for Meriva®, 33% for GA/MD and 37% for turmeric), increased differently in all ingredients in the transverse tract (from the 34% of Meriva® to the 60% of turmeric), and showed the highest values in the descending colon, ranging from 46% (Meriva®) to 61% (turmeric). Overall, Meriva® displayed the highest stability to microbial biotransformation in each tract when compared to the other ingredients, followed by GA/MD and turmeric.

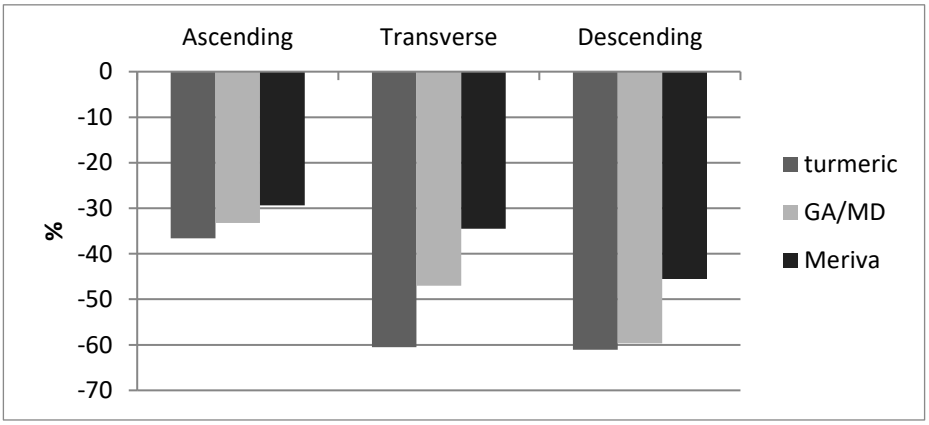

Figure 2. Effect of 5-hour microbial biotransformation on the curcuminoid content of the ingredients. Data are expressed as percent variation of total curcuminoids at t_{300} compared to those at t_0 in ascending, transverse and descending colon.

3.3 Evaluation of food matrix effect on the degradation of curcuminoids along gastro-intestinal tract

To evaluate the food matrix effect on the degradation of curcuminoids along the gastro-intestinal tract, the yogurt and the rice enriched with ingredients (R-GA/MD, R-turmeric and Y-GA/MD) underwent the same protocol of simulated digestion. The enriched yogurt and rice were analyzed within 5 days from when they were formulated although by previous analyses, the curcuminoids resulted stable up to 30 days in the enriched yogurt. This step did not affect the curcuminoids concentration.

The results showed in **Table 4** highlight how the matrix yogurt protected better the curcuminoids from the degradation mediated by the gastric environment (-10%) compared to the matrix rice (-17% for R-GA/MD and -19% for R-turmeric), but these differences disappear after the digestion in the small intestine.

Table 4 Effect of an *in vitro* gastro-intestinal digestion on curcuminoid content in the foods. Values are expressed as µg of total curcuminoids per gram of digested food. Percent variations of digested sample compared to the undigested are reported in brackets. Different letters represent significant differences within the same sample (column) ($p<0.05$).

	R-GA/MD	**R-turmeric**	**Y-GA/MD**
Undigested sample	418±27 [a]	426±64 [a]	478±17 [a]
Gastric phase	347±8 [ab] *(-17%)*	344±26 [ab] *(-19%)*	429±29 [a] *(-10%)*
Intestinal phase	304±6 [b] (-27%)	289±25 [b] *(-32%)*	358±41 [b] *(-25%)*

The results of bioaccessibility (**Fig. 3**) showed a curcuminoids content higher in the soluble fraction of rice samples (6% and 9% for R-turmeric and R-GA/MD respectively) compared to yogurt (1.3%), with a bioaccessibility about 7 times higher for R-GA/MD when compared to Y-GA/MD.

Moreover, the solubility of curcuminoids increased when the ingredients were added to the rice samples compared to when they were evaluated alone; particularly, the bioaccessibility increased of 3 times for GA/MD, and of 30 times for turmeric.

These results demonstrated the positive effect of the rice matrix on the bioaccessibility of curcuminoids. A similar result was observed in a clinical trial by Vitaglione et al. (2012), who suggested that the curcuminoids encapsulated with cellulose derivates and vegetable oil and added to bread, showed a greater absorption compared to the non-encapsulated. The bioaccessible fraction of Y-GA/MD was instead just the 1.3%, a result not expected considering that, usually, the presence of emulsified lipids (like the fats in our plain yogurt), respect to non-emulsified ones, increases the bioaccessibility of curcumin (Zou et al., 2015).

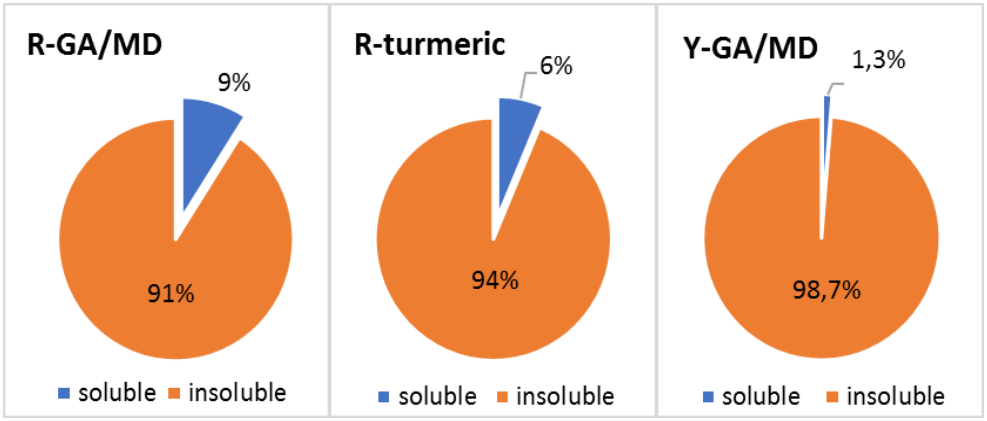

Figure 3. Bioaccessibility of curcuminoids after *in vitro* gastro-intestinal digestion of foods. Values are expressed as percentage of soluble (bio-accessible) and insoluble (non-bioaccessible) fraction.

The enriched rice, showing the best results in terms of bioaccessibility, was also tested with the SHIME® system. After the pancreatic digestion of R-GA/MD and R-turmeric, the insoluble fraction underwent 5 hours of colonic fermentation in the ascending, transverse and descending tract (**Fig. 4**). As previously observed for the ingredients alone, also for the enriched rice the degradation was lower in the ascending colon than in the other two tracts: the percent decreases were 34%, 44% and 49% for R-GA/MD, and 26%, 52% and 55% for R-turmeric in ascending, transverse and descending colon, respectively.

Overall, curcuminoids were more degraded by the colonic microflora when the ingredient was alone compared to when it was added to a food, confirming an expected protective role of the food matrix.

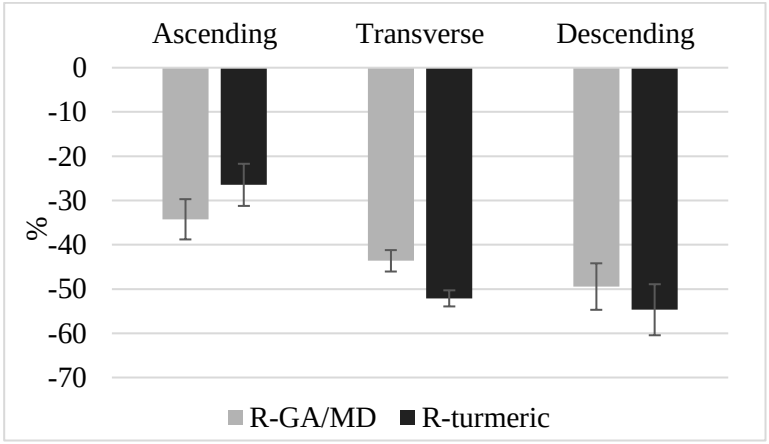

Figure 4. Effect of 5-hour microbial biotransformation on curcuminoid content of the foods. Data are expressed as percent variation of total curcuminoids at t_{300} compared to those at t_0 in ascending, transverse and descending colon.

3.4 Morphological analysis of the microencapsulated ingredient

The ingredient GA/MD was also characterized for the surface and size of microspheres. SEM analysis showed differently sized microspheres often aggregated in agglomerates, a phenomenon probably due to the hygroscopic nature of wall material (**Fig. 5**). The dimensions ranged from 1 to 15 µm: the smallest microspheres displayed a dented and collapsed surface, attributable to the shrinkage of the particles during the drying process, while the biggest ones presented breakages on the surface and seemed to be a collection of more microspheres.

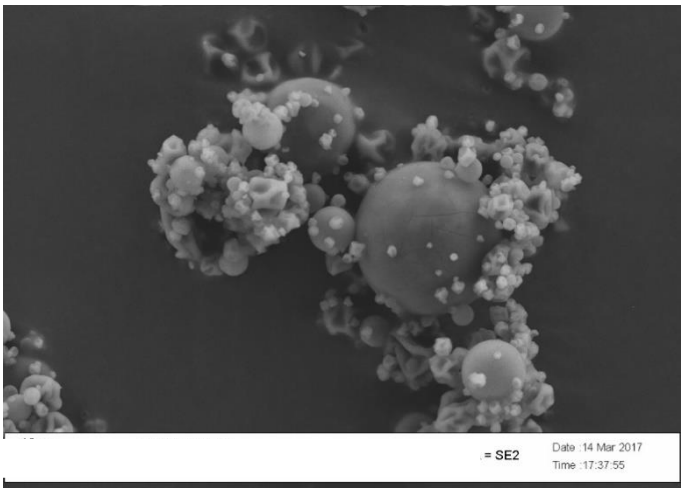

Figure 5. SEM image of the microencapsulated ingredient (GA/MD). Magnification: 5000x.

4 Conclusions

Low bioaccessibility and stability of curcuminoids limits the beneficial properties of turmeric. In this study, we investigated the effect of a microencapsulation with food grade wall materials of turmeric oleoresin on the *in vitro* bioaccessibility and microbial biotransformation of curcuminoids, compared to the non-encapsulated

commercial ingredient (turmeric) and to a patented ingredient for pharmaceutical uses (Meriva®).

The curcuminoids were significantly degraded to the gastro-intestinal conditions in all formulations. However, their *in vitro* bioaccessibility in the microencapsulated ingredient resulted 15-fold higher than the one of turmeric powder and for the rice enriched with the spray-dried ingredient about 3- and 2-fold higher respect to the ingredient alone and the Meriva, respectively.

Considering the suggested ADI of curcumin and the results of in vitro bioaccessibility, an amount of 0.5-1.3 g of GA/MD alone or 0.17-0.4 g of GA/MD added to rice, might be enough to reach the ADI value.

Besides, the rice matrix confirmed to protect the curcuminoids from their degradation that occurs during the transit in the three tracts of colon by microflora.

In future, other studies will be needed to evaluate the performances of the microencapsulated ingredient formulated in other food matrices, like those containing phospholipids (eggs or vegetable oils). Moreover, the contribution of other food grade wall materials to the gastro-intestinal stability of the main compounds should be studied as well.

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6. Discussion

The increased scientific evidences about the beneficial properties of certain foods or food compounds, as the polyphenols, as well as the research for longevity, have risen the request for well-being/functional foods, able to promote health and reduce the occurrence of diseases. To understand the global size of functional foods market, it is enough to comment that, in 2016 only, they generated revenues for 70 bilions of dollars (Functional Foods Market Report 2016-2026).

Among the technological processes currently employed for the development of ingredients for health foods, such as the extraction of valuable compounds, the fermentation, the fat reduction or the enzymatic transformation, there is also the microencapsulation by spray-drying.

The microencapsulation is largely used in the food and agriculture areas, because it is cheaper and easier than the nanoencapsulation, primarily chosen for medical purposes. Moreover, the nano-based materials are currenlty not welcomed in Europe, because they are considered “novel ingredients”. Besides, spray drying is the most predominant microencapsulation process in food field, because it is economically attractive, widely available and offers excellent protective performances.

The application of the spray-drying technique for food purposes, in fact, may present various limitations. Primarily, the choice of wall material (a crucial step representing the selection of the best performing material) is often limited to water soluble materials, depending on the equipment generally used by food companies: differently from the pharmaceutical ones, these plants are not suitable to atomize organic solvents or alcohols in high percentage. The atomization temperature may represent a problem for the encapsulation of heat unstable compounds. In case of aqueous dispersions, the inlet temperature must be higher than 100 °C, because it must provide the sufficient latent heat to allow the evaporation.

Consequently, in the era of functional foods, including those enriched in phytochemicals, food industries have high expectations regarding the application of spray-drying to healthy ingredients, which are often ingredients rich in molecules highly unstable to environmental and technological conditions. Moreover, consumers are increasingly focused on "natural products", thus the choice of wall materials is also often confined to few *food grade* materials. The "natural driven technologies" fit well also with the concepts of and "sustainable" and "green" chemistry.

Based on these knowledges, this PhD project aimed to develop new polyphenols-based ingredients, exploiting the spray-drying technique, useful for the enrichment of foods in health compounds.

We chose to microencapsulate polyphenols belonging to three different categories and present in three different foods.

The first ones were the polyphenols from cocoa hulls, mainly flavanols such as epicatechin, catechin and oligomeric procyanidins. The idea to recover these molecules from cocoa hulls, a by-product resulting from the processing of cocoa during roasting, is well supported by literature data, confirming the very high concentration of antioxidant polyphenols in this matrix.

In a recent review on cocoa hulls, Okiyama et al. (2017) confirmed that they are an example of promising by-product, not only because they are an important source of fiber, but also for their content in phenolic compounds. The concentration of phenolic compounds in CH depends on several factors, like the variety, the country of origin and the processing operations. Our results showed that the concentration of phenolic compounds, measured by Folin Ciocalteu's method is of 1.1%, similar to that reported by Arlorio et al. (2001), and the most abundant flavanol recovered is epicatechin, similarly to cocoa beans (Bordiga et al., 2015).

The recovery of substances from by-products or waste of manufacturing is a popular procedure to exploit wastes from agrofood chains, as well as one of the aims of the so called "circular economy". In a circular economy, waste is reduced, recycling is promoted, and resources are minimised. Therefore, by reducing food waste, food production decreases its environmental impact and becomes more economically viable. This can lead to a sustainable and competitive economy, characterized by major benefits for Europe, contributing to innovation, growth and job creation (https://ec.europa.eu/growth/industry/sustainability/circular-economy_it).

Moreover, cocoa flavanols demonstrated positive effects on metabolic disorders and cancer prevention, and particularly on cardiovascular diseases, as showed in many papers. In fact, also the European Food Safety Agency (EFSA) confirmed the beneficial properties of cocoa, permitting the use of the health claim "*cocoa flavanols help maintain the elasticity of blood vessels, which contributes to normal blood flow*". Considering that a healthy adult individual should consume at least 200 mg of cocoa flavanols daily to obtain the claimed effect (EFSA, 2012), this could be only covered by a daily intake of a considerable amount of cocoa powder, or dark chocolate. Therefore, the formulation of alternative ingredients with healthy properties represent a valid solution to increase the offer of healthy ingredients.

The second ones were the polyphenols obtained from Artemide rice, a new Italian variety of black rice, rich in anthocyanins, especially cyanidin-3-O-glucoside. Black rice has a higher content of phenolics and a greater antioxidant capacity than white and red one. Even the phenolic profile changes significantly among differently colored rices and among the varieties with the same pigmentation. The anthocyanins are described to be present only in black rice and, for Artemide cultivar, they are more abundant than in Nerone and Venere (Bordiga et al., 2014).

These molecules are healthy because they promote the decrease of the oxidative stress, prevent the inflammation and reduce the risk of developing chronic diseases, like cardiovascular disease, type 2 diabetes and some forms of cancer.

These two categories of polyphenols, flavanols and anthocyanins, were firstly extracted from their respective food matrices, by testing food grade solvents in different ratios. The extracts were analyzed for their polyphenols composition by HPLC, and spectrophotometrically for their total polyphenols content, antioxidant capacity, condensed tannins and total anthocyanins.

The extract obtained with a mixture of water and ethanol 50:50 (V:V) resulted the best one in both cases, confirming the usefulness of water to hydrate and "open" the complex structure of food, allowing the penetration of the organic solvent and consequently improving the extraction of polyphenols.

The mixture of water and ethanol allowed obtaining extractive yields comparable to other extraction methods, where solvents not suitable for food industry were chosen (like a mixture of methanol, water and formic acid, used for the extraction of anthocyanins from Artemide rice by Bordiga et al. (2014), or the methanol acidified with hydrochloridric acid, used by Arlorio et al. (2001) for the extraction of polyphenols from CH).

Moreover, also the extractive process affects the amount of available polyphenols and the type of molecules. For example, the supercritical fluid extraction allows avoiding the use of organic solvents and shows good extractive performances, but it displays some limitations, such as the high costs, the low reproducibility and often the impossibility of scaling it up. Instead, the ultrasound assisted extraction is one of most common procedures to extract polyphenols in laboratory and, increasingly, at industrial level. The ultrasound assisted extraction increases the polyphenol yield and reduces the extraction time respect to the

traditionally carried out maceration, and promotes high extractive yields using GRAS (Generally Recognized as Safe) solvents (Vilkhu et al., 2007).

In preliminary tests, several wall materials, in different concentration with the core material, were tested, but only GA and MD were chosen for the microencapsulation, because these coating agents give more sliding powders. Before the atomization, the ethanol must to be removed from the extracts by a rotary evaporator, to allow the dispersion of the polyphenolic extract in the suspension of wall material and prevent its precipitation. Therefore, MD, GA and a combination of them, in different ratios, were tested as wall materials for the spray-drying.

The atomization was performed at the inlet and outlet temperature of 150 °C and 85 °C, respectively, and with a feed flow of 7 mL/min and an airflow of 40 m^3/h. These conditions were set following preliminary tests, to use the lowest temperature that allowed obtaining dry powders and a good microencapsulation yield. Seven different powders of cocoa hulls polyphenols and five of rice anthocyanins were obtained. Regarding cocoa polyphenols, we tested several combinations of the two wall materials, while for rice anthocyanins we tested in parallel also the freeze-drying process.

Spray dried polyphenols were analyzed both at t_0 and during a period of storage, in order to evaluate both the i) impact of the process and the ii) stability of the powders over time. They were then employed as ingredients in model biscuits, to analyze the thermo-resistance of encapsulated polyphenols.

In these two works we wanted to microencapsulate and compare polyphenols belonging to two different categories, flavanols and anthocyanins, with the aim of obtaining ingredients stable both to time and to baking, tested in a model biscuit at 180 °C for 25 minutes. Furthermore, we evaluated the stability of the polyphenolic component to the spray-drying process.

Regarding the study on cocoa hulls polyphenols, the results highlighted the stability of most microcapsules to the spray-drying process and to storage, for what concerned the polyphenol content and the antioxidant capacity. Particularly, a good stability was registered for the epicatechin, the most abundant flavanols in cocoa, the procyanidins B1 and B2, and the gallic acid. Following these results, the stability of cocoa flavanols to spray-drying technique was determined for the first time. Considering that the parameters used for cocoa flavanols atomization were similar to those used for the atomization of olive pomace polyphenols reported by Paini et al. (2015) (150 °C versus 130 °C as inlet temperature and 7 mL/min versus 10 mL/min as feed flow) as well as the positive results of polyphenols and antioxidant capacity stability (up to 70 days at 5°C for olive pomace polyphenols), these spray-drying conditions should be keep into account for the encapsulation of other polyphenols from other matrices.

Instead, regarding the stability of polyphenols to baking, it was registered only for two powders formulated with a high content of MD: the one obtained using only 80% MD and the one obtained using a mixture MD/GA (80:20, w:w). These results confirmed the protective effect of MD to thermal treatments (Paini et al., 2015) also considering the flavanols class.

Thus, we demonstrated that the polyphenolic content of the ingredient obtained with 80% MD and 20% of dry polyphenolic extract resulted the best powder, given its stability to drying, storing for 90 days and baking.

Even the total polyphenol content of Artemide rice resulted stable to the spray-drying process, although cyanidin-3-O-glucoside, the main anthocyanin in this rice, decreased significantly ($p < 0.05$) in all powders. Regarding the stability to storage of rice polyphenols, the extract spray dried with MD and GA (50:50, w/w) resulted the most stable after 30 days, while all other powders displayed a significant degradation of the phenolic component. Likewise, also Bakowska-Barczak and Kolodziejczyk (2011) did not register significant decreases of

antioxidant capacity and total polyphenols content, especially anthocyanins, in blackcurrant extracts spray-dried with MD during 9 months of storage. However, although the biscuits enriched with the microencapsulated ingredients showed a content of polyphenols significantly higher than the control biscuit, the anthocyanins decreased significantly after the baking. The degradation of anthocyanins to heat treatments, as during the cooking of rice, is temperature-dependent (Hiemori et al., 2009; Zaupa et al., 2015). Only the complexation with β-cyclodextrins has demonstrated an improved stability of anthocyanins when compared to the free extract, both in solution and in solid state. The inclusion complex in solid state remains stable at temperatures ranging from 100 to 250 °C (Mourtzinos et al., 2008).

Therefore, it is possible to highlight that the spray drying process, despite the high temperatures used, did not negatively affect the total polyphenol content of both cocoa hulls and rice extracts, and that the microencapsulation of these kind of molecules improve their storage stability. Microencapsulated polyphenols from cocoa hulls resulted more fit to develop a stable ingredient for bakery products, compared to the rice ones. In future studies, the microencapsulated rice extract could be employed in non-cooked foods, or used as "natural" food colorant and other stabilizing agents, like β-cyclodextrins, should be tested to protect the anthocyanins in baked products.

The third work was performed on turmeric oleoresin, solubilized in ethanol and spray dried with a mixture of GA/MD 80:20 (w:w) to obtain a curcuminoid-rich ingredient. This formulation was selected among others in preliminary tests as it displayed the highest solubility. The first aim of this work was to obtain a yellow ingredient (colorant ingredient), alternative to curcumin (E100), suitable for water-based foods. Besides, it was formulated in a way to avoid the additive labelling as "specialty ingredient", as well as to stabilize the color, which is one

of the downsides of curcumin. The idea to "claean" the label of food eliminating the use of food additives is a key trend in food technology today.

Therefore, this food matrix was spray dried at inlet temperature of 130 °C, because the presence of ethanol in the emulsion allowed a temperature lower than the one set for previous spray-drying. The curcuminoids were then quantified before and after being spray dried, to evaluate their stability to the operative conditions. It emerged that spray drying did not affect the curcuminoid concentration in the final ingredient. Moreover, the main "nutrition-driven" aim of this work was to compare the stability of curcuminoids to gastro-intestinal conditions of the spray dried ingredient with two commercial ingredients ("curcuma powder" and Meriva®), also considering their use in two different model foods (yogurt and rice) enriched with the ingredients. Firstly, the samples underwent *in vitro* gastro-intestinal digestion using a standardised protocol (INFOGEST). After digestion, the resulting sample underwent colonic biotransformation using the SHIME® instrument, which simulate the three tracts of the colon (ascending, transverse and descending). The curcuminoids were analyzed, before and after gastric and duodenal phase, to evaluate their susceptibility to digestive conditions. The outcomes confirmed that they are significantly degraded under these physiological conditions, in all formulations considered in this study. Moreover, this research allowed also the evaluation of the *in vitro* bioaccessibility of curcuminoids, resulted 15-fold higher for the microencapsulated ingredient when compared to the raw turmeric powder, thus confirming the great bioaccessibility of curcuminoids in microemulsions (spray dried ingredient and Meriva®). More particularly, we suggest the combination of the microencapsulated ingredient with rice, because the outcomes confirmed a positive effect of rice matrix on the bioaccessibility of curcuminoids, which increased of about 3 times respect to the ingredient alone. The protective effect of this starch-rich food must to be considered as interesting new data about the

formulation of functional ingredient, opening new perspectives also toward the use of modified starches during the ingredient design. Furthermore, FAO, WHO and EFSA recommend an adequate daily intake (ADI) of curcumin of 0–3 mg per kg of body weight (JECFA 2004, EFSA 2014), meaning that a healthy individual of 70 kg should consume 4 to 10 g of turmeric every day. This is an enormous amount for a spice, but if we consider that the bioaccessibility of the spray dried ingredient (resulted 15-fold higher than the turmeric raw powder, even though the curcuminoids were only 1% in the spray dried ingredient prepared in this work) an amount of 0.5-1.3 g of this formulation would have the same bioaccessibility of 4-10 g of turmeric powder. This outcome is of great interest also considering the chance to use this encapsulated curcumin (from turmeric oleoresin) as base for complex ingredients.

Concerning the experiments performed using the TWIN-SHIME® instrument, carried out in collaboration with the Food Quality and Design Group of the Department of Agrotechnology and Food Sciences of the Wageningen University & Research (Wageningen, The Netherlands), they allowed determining the degradation of curcuminoids in each colon tract, ascending, transverse and descending, after 5 hours of colonic biotransformation. This system was set up inoculating all vessels with the same faecal sample, using two vessels for each tract (Twin-SHIME®) and setting different pH: 5.7 ± 0.2, 6.3 ± 0.2 and 6.8 ± 0.2 for ascending, transverse and descending, respectively. Following the equilibration period, the samples were added in each vessel in a right amount to have the same curcuminoid concentration in each vessel. The results of this experiments showed a degradation of curcuminoids in the ascending colon, but lower when compared to the degradation occurring in the descending and transverse tracts, in all analyzed samples. Moreover, when the ingredient was used to enrich a model food (so in a complex matrix), the curcuminoids were also less degraded by the SHIME® microflora, when compared to the ingredient analyzed

alone, highlighting and confirming a protective effect of the food matrix, as partially expected. Considering the obtained results and comparing the costs related to the production of both spray dried ingredient and Meriva®, it is possible to conclude that the formulation of microencapsulated food ingredients can be a useful strategy for the development of functional foods containing curcuminoids, reducing - or avoiding - the consumption of dietary supplements. Obviously, clinical trials should be performed to evaluate the right daily intake of the spray dried ingredient, in the next future. Furthermore, increased studies confirm the key role of microbiota on the regulation of many functions, such as the development of several diseases as well as the promoting of human health. (Lin et al., 2015).

The diet plays a crucial role on the modulating of gut microbiota, and the intake of compounds like polyphenols, particularly those characterized by high molecular weight, have positive effect on the modulation of colonic microflora. Polyphenols are partially fermented and used beside the fibers and the carbohydrates by microbiota producing metabolites which most probably are responsible for the health effect derived from the consumption of polyphenol-rich foods. Therefore, it is important to continue the investigation on the phenolic metabolites and their biological activities (Cardona et al., 2013).

Finally, it is possible to conclude that the spray-drying process applied to polyphenols, such as flavanols, anthocyanins and curcuminoids, and in the operative conditions considered during this PhD project, is a useful technique to obtain microencapsulated polyphenols to be used as ingredients in enriched foods, with functional properties. Even though it did not efficiently protect the anthocyanins from baking, spray dryed powdered extracts are more convenient for an easier technical handling during processing, and for an increased stability during storage.

In future, other wall materials should be tested to study the stability to longer periods of storage at room temperature (technical general conditions). Furthermore, it would be appropriate to study a way to increase the load of polyphenols, to obtain powders richer in these bioactive compounds.

Future studies will be aimed at studying wall materials able to better protect easily the degradable molecules, as the anthocyanins, and to test microencapsulated ingredients in other foods undergoing different technological processes.

On the other hand, for low bioaccessible compounds, as the curcuminoids, the bioavailability of the suggested formulation should be tested *in vivo*, to understand whether it is possible to produce smaller particles for food applications, using food grade materials and economically affordable technologies, always avoiding the "nano" dimension.

Concluding, the investigation on the development of spray dried polyphenols as ingredients for functional foods must go on, evaluating, in addition to their chemical and technological properties, the impact of this kind of ingredients on the organoleptic and sensorial properties of the food, namely flavour/aroma/taste and coloring properties.

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List of publications

Partecipation at congresses

Posters:

"Analytical validation of HPLC-DAD and LC-ESI-MS methods for the quantification of bioactive compounds in *Gentiana lutea* extracts". F. Travaglia, M. Locatelli, **V. A. Papillo**, M. Bordiga, C. Garino, M. Arlorio, J. D. Coïsson *8th International Symposium on Recent Advances in Food Analysis (RAFA)* 7-10 November 2017, Prague (Czech Republic).

"LC-MS identification of diaminobenzoquinones from (ethyl)vanillin in bakery products". **V. A. Papillo**, R. Negri, G.B. Giovenzana, M. Arlorio *5th Mass Food Day* 11-13 October 2017, Bologna (Italy).

"Microencapsulation of turmeric (*Curcuma longa*) oleoresin: improvement of solubility in water and water-based food systems" **V. A. Papillo**, R. Lamera, F. Travaglia, M. Locatelli, J.D. Coïsson, M. Aquino, M. Arlorio *30th EFFoST* 28-30 November 2016, Vienna (Austria).

"Formation of coloured diaminobenzoquinones from (ethyl)vanillin in bakary products". R. Negri, **V. A. Papillo**, G. del Favero, F. Travaglia, V. Gokmen, D. Marko, J. D. Coïsson, G. B. Giovenzana, M. Arlorio. *XI Italian Congress of Food Chemistry* 4-7 October 2016, Cagliari (Italy)

"Industrial progressive pearling for the production of fibre-rich ingredients from common wheat". M. Locatelli, **V. A. Papillo**, F. Travaglia, M. Bordiga, J. D. Coïsson, M. Arlorio, D. Giordano, A. Gazzolo, A. Reyneri, M. Blandino *6th International Dietary Fibre Conference* 1-3 June 2015, Paris (France)

"Application of progressive pearling process to pigmented wheat cultivars to obtain functional ingredients rich in fibre and antioxidant compounds". M. Locatelli, **V. A. Papillo**, J. D. Coïsson, F. Travaglia, M. Bordiga, M. Arlorio, D.

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"Release of the antioxidant capacity from five plant foods during a multi-step enzymatic digestion". **V. A. Papillo,** P. Vitaglione, G. Graziani, V. Fogliano *3rd International Conference On Food Digestion* 11-13 May 2014, Naples (Italy).

Oral presentation:

"Anthocyanin-rich extract from Italian black rice (var. Artemide) as functional ingredient for bakery products". **V. A. Papillo**, M. Locatelli, F. Travaglia, M. Arlorio, J. D. Coïsson *XI Italian Congress of Food Chemistry* 4-7 October 2016, Cagliari (Italy).

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“La tua Leggenda Personale è quello che hai sempre desiderato fare. Tutti, all'inizio della gioventù, sanno qual è la propria Leggenda Personale. In quel periodo della vita tutto è chiaro, tutto è possibile, e gli uomini non hanno paura di sognare e di desiderare tutto quello che vorrebbero veder fare nella vita. Ma poi, a mano a mano che il tempo passa, una misteriosa forza comincia a tentare di dimostrare come sia impossibile realizzare la Leggenda Personale. Sono le forze che sembrano negative, ma che in realtà ti insegnano a realizzare la tua Leggenda Personale. Preparano il tuo spirito e la tua volontà. Perché esiste una grande verità su questo pianeta: chiunque tu sia o qualunque cosa tu faccia, quando desideri una cosa con volontà, è perché questo desiderio è nato nell'anima dell'Universo. Quella cosa rappresenta la tua missione sulla terra.

L'Anima del Mondo è alimentata dalla felicità degli uomini. O dall'infelicità, dall'invidia, dalla gelosia. Realizzare la propria Leggenda Personale è il solo dovere degli uomini. Tutto è una sola cosa. E quando desideri qualcosa, tutto l'Universo cospira affinché tu realizzi il tuo desiderio”.

Paulo Coelho - L’alchimista

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declares:

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