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**HIGH ADDED-VALUE PRODUCTS FROM INDUSTRIAL
CROP BIOMASS: USES IN THE AGRO-FOOD SECTOR**

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PREFACE

The present PhD project reports the research study carried out mainly at the Council for agricultural research and economics, Research center for industrial crops (CREA-CIN) of Bologna.

The studies were supervised from Prof. Alejandro Hochkoeppler, associate Professor at the Department of Pharmacy and Biotechnology, of the Alma Mater Studiorum-University of Bologna and by Dr. Luca Lazzeri, which coordinates the 'biorefinery' research group of CREA-CIN research center, a group characterized by a multidisciplinary approach which covers expertise in agronomy, microbiology, phytopathology, chemistry, biochemistry and technology. In this field CREA-CIN research center has been involved for more than 25 years in the study of minor non-food industrial oleaginous crops, focused on their agricultural production and the technological valorization of biomasses, including co-products. Among the different Green chemistry production chains CREA-CIN has operated mainly in the biolubricant and biofuel sector and is specialized in the study of natural biological active molecules from agricultural biomasses for the management and defense of horticultural and fruit crops, as an alternative to synthetic phytochemicals. In those sectors CREA-CIN research activities has been focused mainly, but not exclusively, on the industrial crop family of *Brassicaceae* and their peculiar defensive system myrosinase-glucosinolate, known for its biological activity against numerous plant pathogens and pests. CREA-CIN contributed to the isolation, purification and biochemical, analytical and technological characterization of the myrosinase-glucosinolate system components and studied their application in the agriculture sector, for crop management and defense, but also in the food-sector, as ingredients for functional food development.

The reported work is the result of a multidisciplinary project, which also involved other research institutions. Among them, the Centre for postharvest protection and storage of horticultural products (CRIOF), Department of Agricultural Sciences of the Alma Mater Studiorum-University of Bologna, covered an important role for the expertise on various topics related to the postharvest life of fresh fruit and vegetables. The CRIOF group, coordinated by Dr. Marta Mari, has collaborated with the CREA-CIN group on this subject for more than 20 years and also supervised this work for the part dealing with the studies on fruit postharvest disease control and quality maintenance by natural biological molecules produced from the *Brassicaceae* family. Within this subject the study was also improved thanks to the collaboration of the research group of Dr. Katya Carbone of the Fruit Tree

Research Centre of Rome (CREA-FRU). Finally, the last part of the work availed itself of the contribution of the research group of Prof. Marco Biagi, Department of Veterinary Medical Sciences, and the research group of the Prof. Donatella Canistro, Department of Pharmacy and Biotechnology, Alma Mater Studiorum-University of Bologna, which conducted the conclusive trials regarding the applicative uses of protein hydrolysates of vegetal origin.

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ABSTRACT

The aim of this work was the study of the transformation and valorization of industrial crop biomasses into high-value products with different applications in the agro-food sector, in a full biorefinery approach. Defatted seed meals (DSMs), co-products of the oil extraction procedure, from different industrial crops of high economic importance such as *Brassicaceae* (Rapeseed, Carinata et al.) and of *Asteraceae* (Sunflower et al.), were used, such as or after processing. Their biological active compound and protein content were employed to produce bio-based products for agriculture and functional food and feed applications. The bio-active molecules, generated by the myrosinase-glucosinolate system of the *Brassicaceae* family, were applied for the postharvest fruit pathogen control (*Botrytis cinerea*) and quality control in strawberry and kiwifruit: fruits were treated with vapors of allyl-isothiocyanate produced from previously formulated and standardized *Brassica carinata* A. Braun or *Brassica nigra* L. DSM and the pathogen incidence, biochemical and nutraceutical evaluations were performed after fruit storage. Also another brassica DSM, from *Eruca sativa* Mill., was studied for the glucosinolate content, to produce innovative food, as functional bakery products, rich in healthy compounds. Finally the protein component of DSMs from sunflower (*Helianthus annuus* L.) and rapeseed (*Brassica napus* L.) DSMs, were employed for the production of hydrolysates by mild enzymatic hydrolysis. The sunflower hydrolysate produced by a two-step process, showed a good free amino acid content and was tested as biostimulant by *in vitro* and *in vivo* bioassays, showing an interesting hormone-like properties and a stimulating effect on roots. In this context the biostimulant properties derived from another Brassica, *Barbarea verna* Mill. Asch., DSM were also explored in *in vitro* bioassay. Finally sunflower and rapeseed hydrolysates, obtained by a one-step hydrolysis process, resulted as a product rich in small peptides and low amount of amino acids, a characteristic potentially suitable for the development of pet food supplements.

ABBREVIATIONS

ABTS•+	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACO1	ACC oxidase 1
Act d1	Actinidin
ADI	Acceptable daily intake
AFRs	Adverse food reactions
AITC	Allyl-isothiocyanate
APH	Animal protein hydrolysate
AsA	Reduced ascorbic acid
AsAC	Ascorbic acid content
AU	Anson Unit
BAM	Beta-amylase
BBI JU	Bio-based industries joint undertaking
BHA	Butylated hydroxyanisole
BHAE	Butylated hydroxyanisole equivalent
CA	Controlled atmosphere
CAE	Catechin equivalents
CAR-PDMS	Carboxen-polydimethylsiloxane
CAT	Catalase
CHS3	Chalcone synthase
CREA-CIN	Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria – Centro di ricerca per le colture industriali
CV	Cyclic voltammetry
DH	Hydrolysis degree
DHA	Dehydroascorbic acid
DM	dry matter
DNA	Deoxyribonucleic acid

DPPH•	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DPV	Differential pulse voltammetry
DSM	Defatted seed meal
DTNB	5,5'-dithiobis(2-nitrobenzoic acid
EC50	Half maximal effective concentration
EC95	95% maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
EFSA	European food safety authority
ESP	Epithio-specifier protein
ETR2	Ethylene receptor
EU	European Union
FAO	Food and agriculture organization of the United Nations
FDA	Food and drug administration
FID	Flame ionization detector
FLC	Flavan-3-ol content
FMOC	9-fluorenylmethylchloroformate
FPLC	Fast protein liquid chromatography
FW	Fresh weight
GA	Gallic acid
GAE	GA equivalent
GalDH	L-galactose dehydrogenase
GC	Gas-chromatography
GER	Glucoerucin
GL	Glucosinolate
GPP	L-galactose-1-phosphate phosphatase
GRA	Glucoraphanin
GRAS	General recognized as safe

GSH	Reduced glutathione
GSSG	glutathione
GST	Glutathione S-transferase
HPLC	High-performance liquid chromatography
HS	headspace
HSD	Honest significant difference
IAA	Indol-3-acetic acid
IEA	International energy agency
ITC	Isothiocyanate
LAPU	Leucine aminopeptidase unit
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Least significant difference
MDHAR	Monodehydroascorbate reductase
MEA	Malt extract agar
MES	2-(N-morpholino)ethanesulfonic acid
M2PV	1-methyl-2-vinylpyridinium triflate
MRL	Maximum residue limit
MS	Mass spectrometry
MYR	Myrosinase
NADPH	β -Nicotinamide adenine dinucleotide 2'-phosphate reduced
NBT	nitrobluetetrazolium
NSP	Nitrile-specifier protein
OPA	o-phthalaldehyde-3-mercaptopropionic acid
PAL	Phenylalanine ammonia-lyase
PG	Polygalacturonase
POD	peroxidases

PPO	Polyphenol oxidase
PR5	Thaumatococcus-like protein
RMH	Rapeseed meal hydrolysates
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPH	Rapeseed protein hydrolysate
RSD	Relative standard deviation
SAM	S-adenosylmethionine synthase
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SE	Standard error
SME	Medium-size enterprises
SMH	Sunflower meal hydrolysate
SOD	Superoxide dismutases
SPH	Sunflower protein hydrolysates
SPME	Solid phase micro-extraction
SSA	Sulfosalicylic acid
SSC	Soluble solid content
TA	Titrateable acidity
TCEP	Tris(2-carboxyethyl)phosphine
TE	Trolox equivalent
TFP	Thiocyanate-forming protein
TPC	Total polyphenol content
TUA	Alpha-tubulin
UBC9	Ubiquitin conjugating enzyme
UBQ	Ubiquitin
VPH	Vegetal protein hydrolysate

CHAPETER 1.
INTRODUCTION

1.1 The Biorefinery concept in the Circular Bioeconomy

Today more than 80% of energy and 90% of organic chemicals in the world are derived from fossil fuels (oil, coal and natural gas) and their demand is constantly growing due to the world population increase (Maity, 2015). Therefore renewable resources are becoming more and more necessary due to the progressive depletion of fossil supply and their high costs, economic and environmental, linked to the high greenhouse gas emissions and the climate change.

The Bioeconomy is a large and rapidly growing segment of the economy and more strictly of the Green Economy, which policy is centered on the use of renewable raw materials from land and sea – such as crops, forests, fish, animals and microorganisms – and on the application of research, development and innovation and industrial biotechnology to produce added value products as food, feed, biobased-products and bioenergy¹ (Scarlat et al., 2015). The Bioeconomy goals are the reduction of reliance on fossil fuels and non-renewable resources, preventing the loss of biodiversity and limiting soil use transformation, and finally to protect the environment and promote economic growth and local traditions. The scientific research and the biotechnology innovations are the foundation of the related economic activities, where the recycling and re-use of waste in the agro-food, forest, bio-industry and marine sectors, is a part of the winning strategies, promoted by the European community, following the concepts of Circular Economy and environmental sustainability.

According to the REN21's 2016 report only around 20% of global energy consumption comes from renewable energy, of which more than 50% derives by biomasses (biofuels and waste), with China, United States and Germany being the top three countries in renewable power capacities, while Italy is the sixth one (REN 21, 2016). Furthermore, while for energy production other natural sources are also available (wind, sun, water etc.), for the chemical and biotechnological industry, the biomass dependence is even more strict (Kamm et al., 2015). Among the renewable resources, biomasses comprise in fact a large class of materials of organic origin, such as from forest, agriculture and urban waste, including dedicated energy crops and trees, agricultural foods and feed, crop residues, aquatic plants, wood, animal and other waste materials, which can be used as feedstock for the bio-based industry and for bioenergy production (Ohara, 2003). Biomasses availability, sustainable technology progress, in terms of technical performances and cost effectiveness, are necessary for a transition to Bioeconomy (Scarlat et al., 2015). Global Bioeconomy policies and strategies promote the use of dedicated, purposely developed biomasses in a sustainable production system throughout a cascading approach of biorefinery. This means that

¹ <https://ec.europa.eu/research/bioeconomy/index.cfm>

the biomass could be firstly processed into high value products (e.g. pharmaceutical materials, chemicals) and the residues could be then re-used in order to reduce final waste accumulation (Van Lancker et al., 2016). The relevance of these subjects is demonstrated by their strong insertion in the European framework program for research and innovation for 2014/2020, Horizon 2020 (Societal challenge 2: Food security, sustainable agriculture and forestry, marine and maritime and inland water research and the Bioeconomy). In this context in 2014 a public-private partnership between the EU commission and the bio-based industries consortium was officially established (EU Council Regulation No 560/2014 of 6 May 2014), the so called Bio-based Industries Joint Undertaking (BBI JU). This partnership, is focused on the use of biomasses (including their co and by products) for the production of innovative added-value bio-based materials and fuels applying the biorefinery approach as the most effective strategy². However the interest is shared also by other countries, in a world common perspective. In April 2012, in fact, the US administration launched the “National Bioeconomy Blueprint”³, focusing on the bioscience research, and other countries as Japan, India, Brazil, and China are investing in a more sustainable economy (Van Lancker et al., 2016).

The concept of biorefinery, originated in the late 1990s, has been defined by the International Energy Agency (IEA, 2015) as ‘the sustainable processing of biomass into a spectrum of marketable products and energy’. Initially it mainly involved the ethanol fermentation processes for fuels (Keller, 1996), but it is still under constant evolution and growth, driven by the scientific research and technology improvement. Biorefineries are currently classified as 1st to 4th generation based on the applied technology and feedstock raw materials. 1st generation biorefineries process agricultural biomasses as vegetable oils or sugars from food crops for biofuel production; the 2nd generations start from non-food materials, as lignocellulosic biomass, wood, agricultural residues and purpose-grown energy crops on marginal lands; the 3rd generation biorefineries regard the uses of aquatic biomass i.e. algae, while the 4th is under definition and deals with new technologies and biotechnologies, as those called “synthetic biology” for the production of photo-biological solar fuels and electro-fuels (Aro, 2016; Parada et al., 2016). While the application of the 1st generation of biorefineries is now often discouraged owing to their negative impact on food security and supply, the other biorefinery generations are still under development and the 3rd and even more the 4th are currently subjects of many scientific researches (Popp et al., 2014). Nevertheless 1st generation biorefineries could still be considered at local scale in small plants for instance for vegetal oil production directly on the farm (Spinelli et al., 2013; Lazzeri et al., 2015). Finally, the

² http://ec.europa.eu/research/press/jti/factsheet_bbi-web.pdf

³ <https://obamawhitehouse.archives.gov/blog/2012/04/26/national-bioeconomy-blueprint-released>

biorefinery could be projected on an existing abandoned industrial infrastructure with the aim of recovering areas fallen into disuse (*browfield*), or new built (*greenfield*) or could result from an integration of new technologies within existing operating plant structures (*retrofit*) (Batsy et al., 2013).

Agriculture is one of the macro-sectors in which Circular Economy and Bioeconomy strategies are investing most, since a huge quantity of biomass is generated and represents one of the most abundant and low cost renewable resources (Santana-Méridas et al., 2012). Agriculture biomasses include non-edible crop-based residues, as those derived from the field or horticultural production (e.g. cereals, tubers, legumes stalks, wheat straw, leaves, roots etc.) and processing-based residues, derived from the post-harvest agro-industrial processing of the crop and the relative by-products (e.g. husks, seeds, bagasse, peels, hulls, meals from the industry of fruit, coffee, cereal, grape, oil, wood etc.). Great is the economic and social interest in the efficient recovery of agricultural industry residues to be used as a source of energy or as raw materials for the conversion into different bioproducts (animal feed, compost, panels, paper, biofertilizers, biofibers etc) or for the isolation of bioactive natural products through chemical or enzymatic transformation and fractionation technologies (flavonoids, polyphenols, tocopherols, tannins, carotenoids, vitamins, sugars, essential oils, terpenes, lignin, fibre, proteins, peptides, etc).

In the recent years some controversies have arisen from the use of crop biomasses concerning the competition for land, food, feed or sustained carbon storage. For this reason the re-use of marginal, unused or contaminated land and the biodiversity maintenance have been now strongly encouraged, together with the individuation of dedicated local species at low impact, the recycle of crop residues and waste and the improvement of transformation technology efficacy, as valid alternatives to the direct landfilling, according to the Directive 1999/31/EC.

1.2 *Industrial oleaginous crops*

Oleaginous crops are widely used for vegetable oil production for food and non-food applications as bioenergy or industrial green chemistry. They include conventional crops, such as sunflower (*Helianthus annuus L.*) and rapeseed (*Brassica napus L.*), but also other industrial crops form the *Brassicaceae* family (*Crambe abyssinica Hochst.*, *Brassica carinata A.Braun*, *Brassica nigra L.*, *Brassica napus L.*, *Camelina sativa L. Crantz*). They are usually short rotation crops, with a high yield in term of seed and residual biomass, and some of them, as *Brassica carinata*, offer the advantage of being of high rusticity, pedo-climatic adaptability and at low-input request, thus suitable for a sustainable cultivation in marginal unused lands (Lazzeri et al., 2010; Taylor et al., 2010).

The seeds can be considered as a natural chemical factory, starting point for the production and isolation of different bio-based products, fuels, chemicals, cosmetics and pharmaceuticals, from a circular/zero waste Bioeconomy perspective.

The oil fraction represent the 20 to 50 % (w/w) of the industrial culture seed and it was mainly produced for biofuels (e.g. energy crops for biodiesel) and for technical oil. Extraction is usually performed by hexane and/or physical pressing of seeds, the latter determining a higher residual oil content in the press-cake (10-12%). In function of the different physical and chemical properties it could be exploited as primary renewable source in diversified field for the production of biolubricants, biopolymers, hydraulic fluids, cosmetics, bio-plastic, carboxylic acid as building blocks for bioactive molecules, technical media in agriculture (mulching film), bio-construction (Lazzeri et al., 1994; Bergman and Flynn, 2008; Soni and Agarwal, 2014; Li et al., 2015).

The uses of oil have been extensively studied, while other seed components, as the protein fraction and bioactive molecules, have often been neglected.

The residual meal after oil extraction, the defatted seed meal (DSM), constitutes the major by-product (around 60% of the seed) of the process and it is usually used for animal feed. Nevertheless it represents a source of high added-value materials for the agro-food and industrial sectors and its transformation into co-products, in an integrated biorefinery framework, indeed makes it possible to decrease the amount of waste and to increase the efficiency of the entire industrial process. The DSM is mainly composed of proteins (30-40%), fibres (lignocellulosic materials, 10-50%), and minerals (5-7%). The use of plant storage proteins in food and non-food industries is determined by their functional properties which can also be improved by chemical, physical or enzymatic treatments (Santana-Méridas et al., 2012). They could be exploited for example for the production of bioplastics and biopolymers, including packaging materials, emulsifier and surfactants as renewable and biodegradable alternative to petroleum synthetic-based chemicals (Zhang and Mittal, 2010). In animal and human nutrition they could be an alternative protein source to meat proteins, as such or after hydrolysis, which permit to obtain free amino acids and small peptides, with potential biological health promoting properties (Xue et al., 2009; Aoife et al., 2013 and Section 4.2.2). Small peptides could in fact be potential ingredients of functional foods, nutraceuticals, bio-preservatives or pharmaceuticals, with antimicrobial, or antihypertensive properties (Carrasco-Castilla et al., 2012; Wanasundara et al., 2016). Other uses of protein hydrolysates are those within the agricultural field such as biofertilizer and biostimulants for plants (Ertani et al., 2009, Section 4.2.1.). In addition other low molecular weight compounds, in low percentage (3-7%), but with high biological and pharmaceutical value, could be present in the seed, as glucosinolates (Chapter 3),

vitamins, tocopherols, phytosterols and phenolic compounds. They could be used in the food industry for their antimicrobial and/or antioxidant activities, as natural preservatives or as protecting agents against many degenerative diseases as cancer, cardiovascular and neurodegenerative or inflammatory diseases, (Santana-Méridas et al., 2012; Terpinic et al., 2012; Szydłowska-Czeraniak, A., 2013; Das et al., 2014; Dua et al., 2014).

Finally, in order to succeed in applying a valuable biorefinery strategy, biomass proper individuation and selection have to be combined with efficient, innovative, cost-effective and environmentally-friendly processes for the extraction and fractionation of the bioactive molecules from the biomass. Green chemical technologies that take advantage of ultrasounds, microwaves, supercritical fluids or enzymes, the so called White technologies, have to be used with the aim of reducing solvent consumption, to preserve biological activity of the isolated molecules and to define the overall process as environmental sustainable.

1.3 The Bioeconomy in Italy

In Italy, in 2015, the bioeconomy comprises the ‘agriculture, forestry and fishery’ sectors, which amounts to around the 23% of total turnover, the ‘food industries, beverages and tobacco’ sector which reaches the 51,5%, while the bio-based pharmaceutical and chemical industry and the bioenergy and biodiesel production amount for the 3% and 1% respectively⁴.

In the bio-industry sector Italy has a relevant role and important private investments made it possible the realization of biorefineries integrated in the local area which use renewable raw materials or dedicated sustainable crops, for the production of bioplastic and biochemical, revitalizing abandoned industrial sites, unused or no longer competitive (Ganapini et al., 2013). Indeed different projects are recognized as lighthouse projects in Europe by the BBI JU such as the First2run case study at Biocirce⁵, regarding the sustainability of a local integrated biorefinery for the production of biobased products from low input oil crops (e.g. cardoon and safflower).

The rural diversification, the rich biodiversity and the long and rich cultural tradition are in fact prestigious and characterizing elements that make Italian agriculture internationally competitive even on non-food application.

Furthermore the Italian contribution to the bio-based economy is demonstrated by the recent institution of national clusters, partnership of public and private stakeholders companies and research centers, as the National Technology Agrifood Cluster CL.A.N., active in the agri-food

⁴http://www.agenziacoazione.gov.it/opencms/export/sites/dps/it/documentazione/NEWS_2016/BIT/BIT_EN.pdf

⁵<http://www.first2run.eu/>

sector, and the National Technological Cluster of “Green Chemistry” SPRING with the mission of revitalizing the Italian green chemistry value chain, starting from renewable resource from agriculture, for the development of innovative bio-based industries through the promotion of biorefineries integrated on the territory and the implementation of investments in new technologies to stimulate bioeconomy at a national and regional level⁶.

In this contest the Italian VALSO project (technology integrated system for the valorization of the by-products derived from the biodiesel production chain) coordinated by the CREA-CIN research center of Bologna, was funded by the Italian Ministry of Agriculture and Forestry (MiPAAF) in 2010, and, in line with the European and international biorefinery approach, involved private companies, public research centers and universities. The project aimed at valorizing the economic and environmental sustainability of biodiesel chain through the recovery and valorization of by-products, mainly glycerin and defatted seed meals as secondary biomass residues, derived from Italian oil extraction plants. The project worked starting from seeds of the most common sunflower and rapeseed, but also of the promising cultures *Brassica carinata* and *Brassica nigra*. The research project, ended in 2014, permitting the identification of new biobased products, mainly for the industry (biopolymers, hydraulic fluids, coolants), for the agriculture, as technical means (fertilizers, amendments, biostimulants, herbicides, biopesticides), and for animal feed (pet food), that could substitute conventional fossil source chemicals (Lazzeri et al., 2015). The re-use of byproducts and coproducts and the consequent improving of the economic feasibility of the Italian biodiesel chain has been an example of a multidisciplinary, integrated project, whose organization and management could be easily transferred to many other innovative agricultural and food chains with industry involvement. The project was developed following the concept of diversified biorefineries integrated in the local area, linked to the specificity agricultural productivity, by using local raw materials and protecting local biodiversity, key factors for the realization of a Circular Economy and its enhancement of markets and competitiveness.

The CREA-CIN is also partner of the Italian project SUSCACE (Axbb, *Materie prime Agricole italiane per Bioprodotti e Bioenergie* – 2012-2018) focused on the agronomic and productive evaluation of some high-rusticity oleaginous crops (*Linum usitatissimum L.*, *Camelina sativa L. Crantz*, *Carthamus tinctorius L.* and *Crambe abyssinica Hochst.*) for the recovery of raw materials, oil, defatted seed meals and field residues, to be used in the biolubricant, nutraceutical, cosmetic and construction sectors⁷. In April 2016, the research center has being also involved in the five year national project AGROENERGY (D.D. n. 26329, 1 april 2016) -

⁶ <http://www.clusterspring.it/home-en/>

⁷ <http://www.chimicaverde.it/progetto-axbb/>

<http://agroener.crea.gov.it/>), funded by MiPAAF, dealing with the use of agro-forestry and agro-industrial by-product (in the biogas chain) and the use of dedicated cultures (*Camelina sativa L.* and *Carthamus tinctorius L.*) for the biofuel and biolubricant extraction within a biorefinery approach⁸.

The research activities reported in the present thesis have being partly performed under the above mentioned VALSO, SUSCACE and AGROENERGY projects.

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⁸ <http://agroener.crea.gov.it/new.html>

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CHAPTER 2.

AIMS OF THE STUDY

The aim of the thesis was to study the potential uses of industrial biomasses derived by oleaginous crops, in particular the residual meal after oilseed defatting procedure, for the production of high-added value materials for application in agriculture and food and feed sectors.

Defatted seed meals (DSMs) from *Brassicaceae*, *Brassica carinata* A. Braun and *Brassica nigra* L., were mainly examined for the production of the bioactive molecule allyl-isothiocyanate, with the following objectives:

- to develop a rapid analytical method for the screening of the allyl-isothiocyanate releasing properties of biofumigant materials for crop protection and their standardization;
- to apply the gaseous allyl-isothiocyanate in the postharvest phase and to investigate the effect on pathogen control and fruit quality, through the evaluation of biochemical and physiological parameters, in strawberry and in kiwifruit.

DSM from the brassica *Eruca sativa* Mill. was also explored as potential ingredient in functional food for human nutrition, thanks to the healthy properties of the glucosinolate content and the corresponding isothiocyanate production.

Finally, the protein fractions of other DSMs were studied and the production processes of two hydrolysates from sunflower (*Helianthus annuus* L.) and rapeseed (*Brassica napus* L.) DSMs, with different amino acid and peptide compositional characteristics, were set up. The obtained products were preliminarily tested as follows:

- the sunflower DSM hydrolysate was tested as biostimulant for agriculture in *in vitro* and *in vivo* bioassays; in this context the potential biostimulant properties of *Barbarea verna* Mill. Asch. DSM were also investigated;
- both sunflower and rapeseed DSM hydrolysates were tested in animal feed, as supplement for pet diets, evaluating the *in vitro* digestibility test and *in vivo* safety.

CHAPTER 3.

***BRASSICACEAE* DEFATTED SEED MEALS**

3.1. Introduction

3.1.1. The Brassicaceae family

Among industrial crops *Brassicaceae* (Cruciferae) represent a wide botanical family belonging to the Brassicales order that comprise well known plants, historically part of the human diet and widely cultivated as important vegetable, condiment, oilseed, and forage crops (Fenwick et al., 1983). They are composed of 350 genera and about 3500 species, including some edible and industrial oilseed crops of great economic importance (Fahey et al., 2001). Rapeseed (*Brassica napus* L.) for instance, is the most important source of vegetable oil in Europe and the second most important oilseed crop in the world after soybean (Szydłowska-Czerniak, A., 2013). Almost all rapeseed production in EU has shifted to ‘double-low’ or ‘double 00’ or ‘canola’ rapeseed cultivars, with low content of potential anti-nutritional compound as erucic acid in the oil (<2%) and glucosinolates in the meal (< 30 $\mu\text{mol g}^{-1}$), developed by breeding and now mainly used in food and feed production. In addition, it has become in Europe the primary feedstock for biodiesel production and for other non-food applications. Examples of *Brassicaceae* include also *Brassica juncea* L., *Sinapis alba* L. and *Brassica nigra* L. respectively indian, white and black mustard, known as mustard condiment crops (Warwick, 2011) and other edible plants. These plants are often present in human diet and are well-known for their nutritive and healthy properties in folk medicine, but also in the modern medicine, being object of numerous recent research studies (Jahangir et al., 2009). Among those *Brassicaceae* some are broccoli, cabbage, cauliflower, Brussels sprouts, kale (*Brassica oleracea* L.), turnip (*Brassica rapa* L.), radish (*Raphanus sativus* L.), horseradish (*Armoracia rusticana* P.), salad rocket (*Eruca sativa* Mill.), consumed worldwide. The *Arabidopsis thaliana* also belongs to the *Brassicaceae* family and it is well-known as model organism for the cellular and molecular biology research. Conversely wallflower (*Erysimum pseudoraethicum* L.) and honesty (*Lunaria annua* L.), are grown as ornamental flowering plant, while *Isatis tinctoria* L. is an invasive wild species used for producing a blue dye mainly from the leaves and some others plant parts, that grow even on marginal lands. In this regard the CREA-CIN Institute of Bologna has one of the most rich collection in Europe of *Brassicaceae* seeds, from wild and cultivated non-food species, which were characterized for their fatty acids and bioactive molecules content, and some of them also tested in full-field, in order to valorize the *Brassicaceae* biodiversity (Lazzeri et al., 2013).

All plants of the *Brassicaceae* family are characterized by the presence of a bioactive endogenous system, being part of the plant defensive mechanism, the myrosinase-glucosinolate system (MYR-GL), particularly concentrated in the seed, where it accumulates during ripening.

3.1.2. *The myrosinase-glucosinolate system*

GLs are secondary metabolites representing around 5% of seed composition and have been subject of several studies over the last half-century, initially for the effects of various derivatives in food and feed sectors due to the undesired pungent flavour and anti-nutritional characteristics and more recently, for their therapeutics and chemo-preventive properties (Fahey et al., 2001; Holst and Williamson, 2004). GLs share a common hydrophilic β -D-glucopyranose residue linked via a sulphur atom to a (Z)-N-hydroximosulfate ester plus a variable hydrophobic aglycone side chain derived from one of eight amino acids. Among the around 130 GLs documented by 2011, the aglycone side chain may be characterised from aliphatic, arylaliphatic or indolic side chains, and it is the part of the molecule that determines the structural molecular variation of GLs (Agerbirk and Olsen, 2012; Blažević et al., 2016). A single GL is often dominant in brassica plant and their GL quali-quantitative composition largely depends on the plant genotype, the plant organ, growth stage, and even on the cultivation conditions (Björkman et al., 2011; Katsarou et al., 2016).

GLs consistently occur in plants in conjunction with the hydrolytic enzyme thioglucosidase hydrolase, commonly known as myrosinase, MYR (β -thioglucosidase, E.C. 3.2.1.147) (Bones and Rossiter, 1996, 2006; Mithen, 2001). Upon biotic (pathogen attack) or abiotic (harvesting, processing or mastication) lesions that determine physical disruption of the plant cell, GLs and MYR come into contact, triggering, in the presence of water (comprise the endogenous plant water), the hydrolysis reaction that releases, starting from GLs, glucose and an unstable aglycone. Finally the aglycone undergoes a fast Lössen rearrangement to form a broad range of degradation compounds, in function of several factors as substrate structure, reaction conditions, in particular pH and the presence of specifier proteins (Hanschen et al., 2014). The isothiocyanates (ITCs) are often the main products when the reaction occurs at 5-7 pH range, while nitriles, epithionitriles, thiocyanate and oxalidinetiones could be formed under different conditions (**Figure 1**).

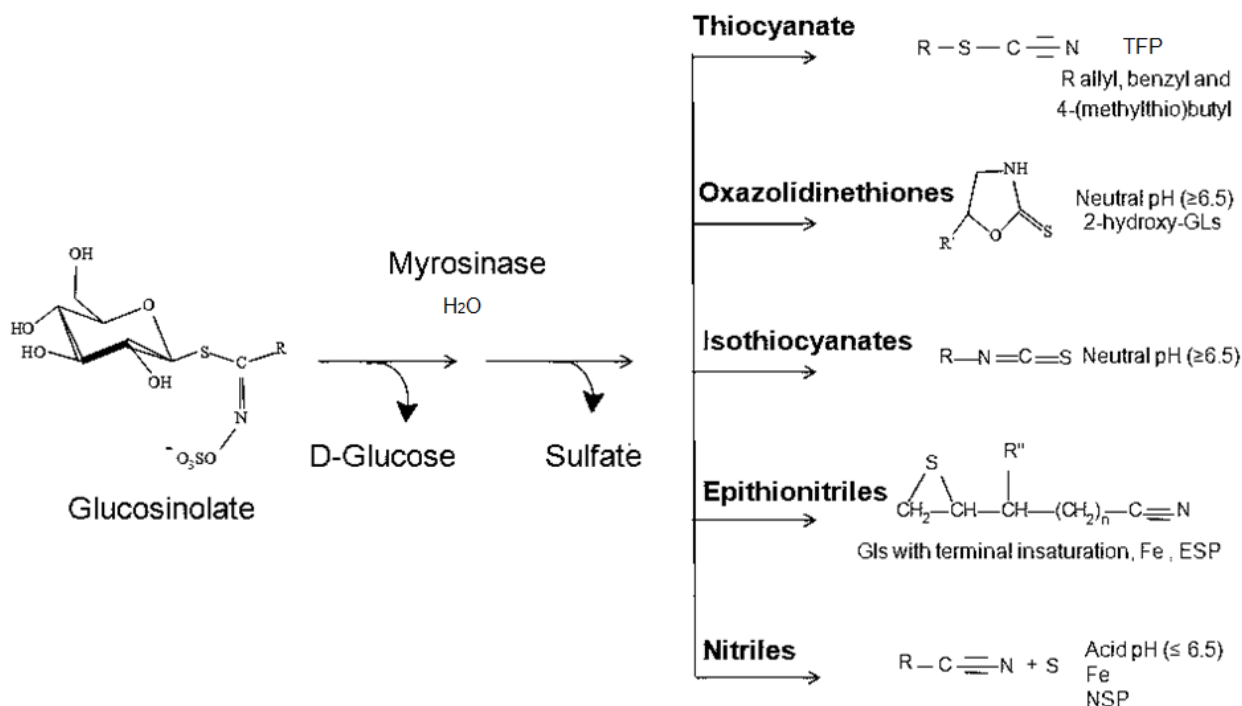


Figure 1. General pathway of MYR-catalyzed hydrolysis of GLs. TFP, thiocyanate-forming protein; ESP, epithio-specifier protein; NSP, nitrile-specifier protein.

The protein cofactors that influence MYR reaction are the heat-sensitive epithio-specifiers (ESPs), nitrile-specifiers (NSPs), and thiocyanate-forming proteins (TFPs), and they redirect the hydrolysis of GLs to nitriles, or epithionitriles or thiocyanates in function of the GL side chain structure and the reaction conditions. The aglycone seems to be their substrate, in presence of MYR, but not the intact GL (Burow et al., 2009; Kong et al., 2012). MYR constitutes a group of isoenzymes that are present in specialized ‘myrosin cells’ distributed throughout seeds (**Figure 2**), seedlings, and mature tissues of plants and are glycoproteins containing various thiol groups, disulfide and salt bridges. They often show multiple forms with different molecular weights (135-580 kD), number of subunits (2-12) and a variable percentage of carbohydrate (up to 22.5%), mostly hexoses (Bones and Rossiter, 1996; Ahuja et al., 2015; Bones, 2015). The most characterized MYR isoenzyme was isolated from ripe seeds of *Sinapis alba*, and consists of two identical subunits with a molecular weight of 71.7 kD, of which 15 kDa are due to glycosylation, stabilized by a Zn²⁺ ion bound on a 2-fold axis, with tetrahedral coordination (Burmeister et al., 1997). MYR activity is enhanced by magnesium chloride and L-ascorbic acid, and it is differently inactivated by temperature and pressure depending on the type of Brassica (Sharma et al., 2012; Dal Prá et al., 2013). MYR also occurs in fungi, bacteria and even in the microflora of soil and of human gastrointestinal tract where converts GLs in nitriles and ITCs, even when the plant enzyme

is inactivated viz. after cooking (Mullaney et al., 2013; Martinez-Ballesta et al., 2015). Anyway gut GL hydrolysis in absence of plant MYR is poor, unless microbiota is previously exposed for days to GL containing food (Angelino et al., 2015).

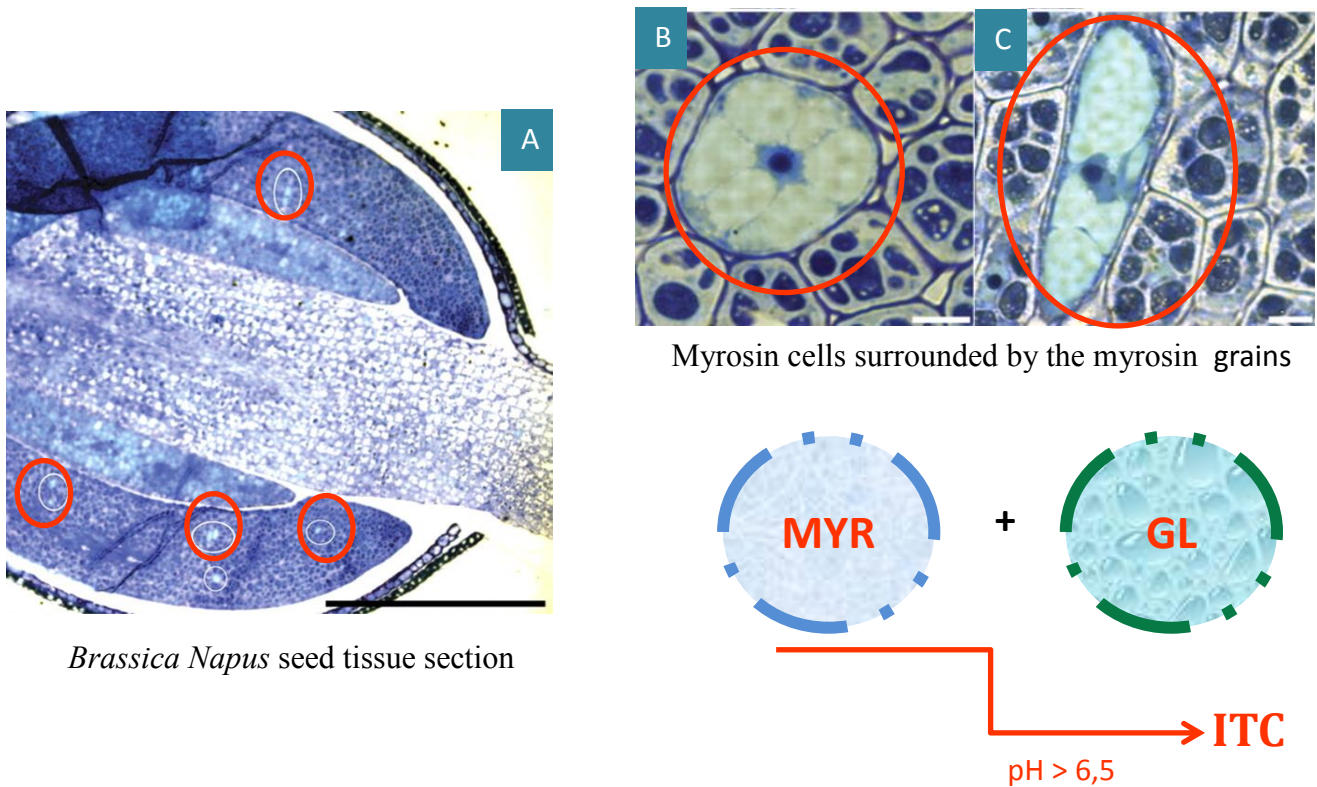


Figure 2. (A) Compartmentalization of the enzyme myrosinase (MYR) in myrosin cells in a *Brassica Napus* seed tissue section, stained with toluidine blue and observed under a light microscope. (B and C) Myrosin cell surrounded by myrosin grains visible as globular green vacuoles, containing MYR. The glucosinolates (GLs) are indeed dissolved in the cytoplasm of neighboring cells. It is only the simultaneous damage of at least three membranes which permits the initiation of the hydrolysis of GLs. Picture rearranged by Ahuja et al. (2011).

ITCs are bioactive compounds largely known for their broad-spectrum biological activity against nematodes, soil-borne fungi and insects or weeds. (Fahey et al., 2001; Matthiessen and Kirkegaard, 2006; Lazzeri, et al., 2009; Lazzeri, et al., 2013). Their biological activity is due to the high electrophilic carbon atom of the ($-N=C=S$) group, which reacts with amines, thiols and hydroxyls to generate thiourea, thiocarbamates and carbamates respectively, thus leading to protein and enzyme biochemical behaviour and biological activity modifications (Dufour et al., 2015). Anyway the mechanism of action at molecular level in cells has not being completely clarified yet (Lin et al., 2000). In bacteria ITCs seem to have a multi-targeted mechanism of action, affecting

several metabolic pathways, probably involving membrane damage, inhibition of enzyme activities, such as those of cellular respiration, induction of heat-shock and oxidative stress responses, and free amino acids depletion (Luciano and Holley, 2009; Dufour et al., 2015). In addition, conjugation with glutathione (via glutathione-S-transferase enzyme) is a major metabolic route of ITC accumulation and detoxification in both rodents, humans and probably also in microorganisms and plants (Øverby et al., 2015; section 3.3). Their different chemical structure indeed determines important chemical and physical properties as hydrophobicity and volatility, but also their specificity towards different organism or different life cycle stages among the same organism (Brown et al., 1997; Smith and Kirkegaard, 2002).

3.1.3. *The Biofumigation*

Crop diseases caused by pathogen and parasite attack are one of the major responsible for product loss and consequent lower income for farmers. Synthetic pesticides and in particular fumigants for soil-borne fungi and pests control are compounds with low selectivity and a high environmental impact (viz. halogenated hydrocarbons as methyl bromide, subjected since 2005 to phase out due to its negative environmental impact). These products are till now the most applied defence strategy in conventional agriculture even if the EU is giving great attention to less impact alternatives. In fact, together with awareness of the toxicity of synthetic compounds and the arising interest for innovation in organic agriculture, there is the need for more environmental friendly alternatives to synthetic pesticide. One of the most promising alternative strategies has been the incorporation of plant tissues or their derived products in the soil, a technique that combines the positive effect of organic matter and biocidal natural molecules (allelochemicals). The use of ITCs generating from brassica plants and products as biological active green manure is one of the most studied control method to replace the use of synthetic pesticide: the so-called biofumigation technique (Kirkegaard and Matthiessen, 2004; Lazzeri et al., 2004; Matthiessen and Kirkegaard, 2006; Gimsing and Kirkegaard, 2008). Recently at the CREA-CIN of Bologna, different liquid and solid formulations were studied applying patented industrial technologies to *Brassica carinata* A. Braun (Ethiopian mustard) defatted seed meals (DSMs), and these biofumigant products are today largely utilized in organic and conventional horticulture as pre-plant treatment, but also for the plant defence and control during cultivation and, although still at experimental stage, for the fruit and vegetable pathogen control in postharvest phase (Lazzeri et al., 2010, 2013; Section 3.2 and 3.3). Allyl-isothiocyanate (AITC) is the degradation product of sinigrin (2-propenyl GL) present in the DSM as basic component of those products, and it is a volatile reactive molecule with favourable

chemical-physical properties (as the good volatility), that results in a broad-spectrum biological activity (Lazzeri et al., 2009, 2011; Benfatto et al., 2015; Section 3.2).

3.1.4. *Brassica beneficial properties in human health*

ITCs are known and recently studied also for their chemio-protective and antioxidant activities assayed *in vitro*, and *in vivo* in human and in epidemiological studies (Herr I. and Büchler, 2010). The glucoraphanin, 4-methylsulfinylbutyl GL, found in broccoli, precursor of the ITC sulforaphane, has been the focus of many of these studies, but there are other ITCs with interesting potential health promoting properties. Their anti-carcinogenic activity is due to direct detoxification of xenobiotics, as heterocyclic amines, by inhibition of phase I enzymes of the cytochrome P450 system, responsible for the activation of numerous carcinogens. In addition, ITCs are potent inducer of numerous nuclear factor erythroid-derived 2 (Nrf2)-dependent phase II enzymes involved in xenobiotic detoxification (NADH quinone reductase, NQO1, γ -glutamylcysteine synthetase, GGCS, heme oxygenase-1, HO-1, glutathione S-transferase, GST, glucuronosyl transferase, epoxidic hydrolases). They are characterized from an inhibitory activity towards inflammatory cytokine production (iNOS, COX-2, IL-1 β and TNF- α) and they suppress the signal transduction mediated by the nuclear factor NF- κ B involved in the production of adhesion molecules, inflammatory cytokines, growth factors and anti-apoptotic factors. ITCs are thus indirectly involved in the elimination of reactive anti-oxidants (ROS). They also induce cell cycle arrest and apoptosis in cancer cells (Melchini and Traka, 2010; Sulforaphane Glucosinolate Monograph, 2010; Zhang, 2012; Gupta et al., 2014). They conjugate with intracellular glutathione and could affect the redox homeostasis by depleting it and by inhibiting glutathione/thioredoxine reductases (Hu et al., 2007; Zhang, 2000). Anyway, the ITC biological effects are dose-dependent and so they can become toxic at high concentrations, hence, bioavailability is a fundamental key factor for exerting beneficial properties. Once absorbed by the intestine they are metabolized in the liver to N-acetyl cysteine conjugates and finally excreted in urine as mercapturic acids (Conaway et al., 2002). In addition to their antioxidant and anti-carcinogenic activities they also exert their health effect throughout a broad range of anti-microbial activity against several human bacteria, in particular the *Helicobacter pylori* (Fahey et al., 2013). Allyl-isothiocyanate as well, known above all for its broad-spectrum antimicrobial activity, seems to be a good candidate as a cancer chemo-preventive agent, in particular acting in the bladder, where it accumulates after oral exposition, even if at high dose levels exhibit a low degree of cytotoxicity and genotoxicity in animal studies (Zhang, 2010). On the contrary, neoglucobrassicin (1-methoxy-3-indolylmethyl GL) is a potent mutagen and it counteracts some inductive effects of sulforaphane, while progoitrin and epi-progoitrin are considered

goitrogenic (Fahey et al., 2001; Haak et al., 2010). Furthermore the well documented pleiotropic actions of ITCs was recently completed by studies on the detoxifying effect in carcinogenesis of intact GLs (glucoraphanin and glucoreucin), largely supported by clinical study (Abdull Razis, et al., 2010; Yu Jiang, et al., 2014).

Brassicaceae vegetables are also a rich source of other health promoting nutrients and phytochemicals apart from GLs, such as vitamins (carotenoids, tocopherol, ascorbic acid, folic acid), minerals (Cu, Zn, P, Mg, among others), fibers, soluble sugars, amino acids and indole phytoalexins and phenolic compounds. All these biologically active molecules contribute to the antioxidant, anticarcinogenic, and cardiovascular protective activities of Brassica vegetables (Jahangir 2009, Reviews). Among the different phenolic compound categories *Brassicaceae* mostly contains flavonoids, in particular flavonols, although the composition could be extremely different in function of the species and cultivation environmental factors (Cartea et al., 2011). In particular, some Brassicas as *Brassica juncea*, characterised by a high GL content both in root and plant aerial part, are very rich in polyphenol compounds active as anti-diabetics and antioxidants at the brain level with excellent benefic effect on cognitive functions (Thakur et al., 2013). *Camelina sativa* L. Crantz DSM resulted to be a good source of GL degradation products also containing good amount of quercetin, synergically acting as detoxifying agents in hepatic cells (Das et al., 2014). The potentiality of these molecules for human health and protection from cardiovascular and metabolic disease are gaining more and more interest and research is directed towards the study of ‘functional’ and/or ‘enriched’ food with specific benefic effects (Section 4.1).

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3.2. Screening of isothiocyanate releasing properties of biofumigant materials in a simple model system

3.2.1. Introduction

Biofumigation refers to the use of isothiocyanates (ITCs) released from glucosinolate (GL) hydrolysis in *Brassica* crops for the management and defense from soil-borne pests and pathogens control (Kirkegaard and Matthiessen, 2004; Lazzeri et al., 2004). GL secondary metabolites are present in all plant organs of brassica plant, but after seed ripening they are concentrated in the seeds, where they accumulate during seed development. Seeds are also the main source of oil for industrial and bioenergy purpose. The defatting procedure by pressure preserves the high GL content and residual defatted seed meals (DSMs) are therefore an abundant by-product and at the same time a good starting material for the production of innovative biofumigant materials. The DSM offers in fact different advantages if compared to the use of fresh manure commonly used in the biofumigation technique, as its higher availability and low moisture content, makes it an optimum stock material with a stable composition. Nevertheless seed meal oil extraction by pressure plant deactivates almost completely the endogenous myrosinase (MYR) enzyme and the consequent biological activity of the material is compromised. A patented biotechnological formulations of DSM was set up and permitted to restore the MYR-GL system and the ITC release after watering (Lazzeri et al., 2010, 2011). Actually formulated *Brassica carinata* A. Braun (Ethiopian mustard) DSM is the basic component of different commercial bio-products, as well as experimental ones, with different ITC releasing properties. 'Biofence' has been registered since 2004 in the Italian Catalogue of Fertilizers, and today it is admitted in organic farming and is marketed as a fertilizer with soil conditioning effects. *B. carinata* is an industrial crop that is gaining growing interest for its agronomic characteristics, especially in the Mediterranean area, and was thus selected as starting crop for seed and DSM production to be used in formulates. It is in fact an industrial crop with favorable cultivation features, as high productivity and biotic and abiotic stress resistance, high seed erucic acid oil content, mainly used for the biodiesel and other bio-based materials production (biopesticide, plastics, polymers, pharmaceutical, and nutraceutical, etc.) and high biomass production, represented by crop residues (Del Gatto et al., 2015). Instead DSM has a good GLs content ($75\div 140 \mu\text{mol g}^{-1}$ of DSM, depending on the defatting procedures) mainly constituted by sinigrin (2-propenyl-GL) (~96% of sinigrin and ~4% of the hydroxylated indolyl-methyl-GL, the 4-hydroxy-glucobrassicin). Allyl-isothiocyanate (AITC) is the product formed by the MYR catalyzed hydrolysis reaction in presence of water and at 5-7 pH range (Mari et al., 1993). AITC is a molecule characterized by a high vapor pressure

and, especially in vapor phase, has largely demonstrated high biological activity against several plant and soil pathogens (Isshiki et al., 1992; Delaquis and Mazza, 1995).

The optimized release of ITC from Brassica derived products represents a key factor for the achievement of their maximal biofumigation potential (Matthiessen and Kirkegaard, 2006). Therefore a method for rapid screening of those material ITC releasing properties, beside the systematic characterization performed by classical analysis of glucosinolates (GLs), lipids and myrosinase (MYR), is necessary. In the present work a simple model system based on headspace gas-chromatographic (HS-GC) technique, that gives a first insight of ITC kinetic release properties was developed. The method parameters were determined and its potential applications evaluated.

3.2.2. *Material and methods*

Meals

For method development trials, the cultivar *Brassica carinata* ISCI7 was grown in the experimental farm of Budrio (Bologna-Italy), located in the Po Valley (44° 32' 13" N, 11° 29' 40" E., altitude 29 m. asl).

All the cultivation phases, from sowing to harvesting, were performed by low impact cultivation techniques, applying neither irrigation nor chemical treatments. After harvesting, seeds were cleaned, partially dried and milled. Seeds and meals were stored in darkness at 19°C and 40% relative humidity. The meals were totally or partially defatted by hexane at room temperature in laboratory (meal:hexane 1:10 w/v). For Biofumigant material quality control trials, DSMs, defatted by an endless screw press in a temperature and pressure controlled procedure, were provided as pellets by Agrium Italia SpA (Livorno, Italy) and subsequently milled. In this case the MYR-GL system was differently reactivated by a patented procedure (Lazzeri et al., 2010). Meals or pellets were all ground to a 0.5 mm size before analysis and characterised for main components including GL content and MYR activity (Lazzeri et al., 2011; Franco et al., 2016). The MYR assays were carried out with the pH-stat method at 37 °C and one MYR unit (U) was defined as the amount of enzyme that hydrolyses 1 $\mu\text{mol min}^{-1}$ of sinigrin. The GL content was determined by the desulf-GL HPLC analysis following the UNI EN ISO 9167-1:1992/Amd 1:2013 method with some minor modifications and was expressed as $\mu\text{moli g}^{-1}$ of DSM calculated at 0% lipid.

Analytical system and method validation

The selected analytical system was composed by 1 L screw Erlenmayer flask equipped with a pierceable rubber septum where the MYR-GL reaction was activated by the addition of water to

the thin powdered meal at the bottom of the flask. The flask was maintained at 25 °C. AITC was produced in gaseous form by hydrolysis of sinigrin and its quantification was performed by analyzing 100 µl of the atmosphere inside the flask every ten min by an Agilent 7820AC GC system equipped with an Agilent HP-5 column (30 m length, 0.32 mm inner diameter, 0.25 µm film thickness) and FID detector (Agilent Technologies, Santa Clara, USA) (GC-FID). Injector and detector temperatures were set at 200°C and 300°C respectively; the oven program started at 60°C and raised to 120°C at a rate of 10°C min⁻¹; the carrier gas (He) flow rate was 1 mL min⁻¹ and the splitless injection mode was selected. The AITC amount was determined on the basis of a defined ten-point calibration curve using a pure AITC standard (Sigma-Aldrich, Saint Louis, USA). Linearity and validation range, limit of detection LOD and quantification LOQ, were experimentally determined from the calibration curve and precision was evaluated by the calculation of assay variability expressed in term of relative standard deviation % RSD, from six replicates (Sanagi et al., 2009). For the AITC quantification at the final optimal determined assay conditions a calibration curve was also constructed with pure AITC in presence of sunflower DSM as control (0.1 g and 0.75 mL of water), in order to consider the matrix effect of the meal on AITC release. For meal releasing properties evaluation, kinetic curves were drawn by plotting the AITC yield % versus time. AITC yield was calculated on the basis of the DSM sinigrin content determined by classical desulfo-HPLC analysis as described above.

Different meal to water ratios and meal quantity were tested in order to obtain the maximum AITC yield. Measurements were replicated at least three times.

Method applications

The method was applied for the fast evaluation of different parameter effects on AITC production. The meal oil content effect was taken as an example. The kinetic plot was constructed for native *B. carinata* meal with an oil content of 44% and DSMs partially and completely extracted by hexane, with a 32.4 and 3.9% of residual oil respectively.

Biofumigant material quality control

The method was applied for the fast evaluation of MYR-GL system changes brought to different formulates. Deactivated *B. carinata* DSM was the defatted meal derived, such as, from the pressure plant. Different Biofence formulations, developed in order to reactivate the MYR-GL system, were tested and compared. The kinetic plots (AITC µmoles g⁻¹ of DSM vs time) were then constructed for deactivated *B. carinata* DSM, the classical Biofence formulation and some

experimental new products called Fast Biofence, Plus Biofence and Fast-Plus Biofence. Formulation details are not reported as confidential (Lazzeri et al., 2010).

Statistics

Data expressed as mean \pm standard deviation were subjected to one-way analysis of variance ANOVA and Tukey's post hoc test performed with R software (R version 3.00.00, The R Foundation for Statistical Computing) and $P < 0.05$ was considered statistically significant.

3.2.3. Results and discussion

Analytical system and method validation

A calibration curve for AITC headspace quantification was constructed by adding pure aliquots of liquid AITC to the flask, at 25 °C and analyzing the atmosphere by GC-FID (**Figure 1**).

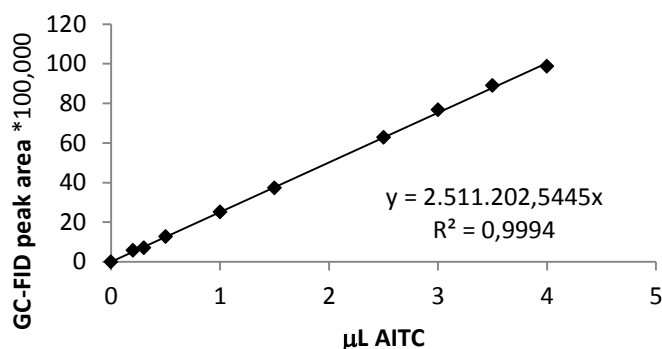


Figure 1. Allyl-isothiocyanate calibration curve determined by headspace-GC-FID analysis in the flask-model system.

A good linearity was established until 4 $\mu\text{L L}^{-1}$ (41.15 $\mu\text{moles L}^{-1}$) and calculated parameters reported in **Table 1**, show the good precision of the analytical method.

Table 1. Method parameters determined with pure allyl-isothiocyanate (AITC), at 25°C.

Method parameters	
Linearity range	0.69 - 41.15 $\mu\text{mol L}^{-1}$
r^2	0.9994
AITC Retention time	4.9 min
LOD	0.69 $\mu\text{mol L}^{-1}$
LOQ	2.29 $\mu\text{mol L}^{-1}$
RSD %	3.18*

*calculated for maximum values

DSM of *B.carinata* defatted by hexane at lab scale used for method development had a GL content of 137,5 $\mu\text{mol g}^{-1}$. First, different quantities of DSM were tested maintaining the meal:water ratio of 1:7.5 (**Table 2**). Good yields were found with 0.1 and 0.05 g, while with 0.2 g the AITC yield % was significantly lower, probably due to the higher quantity of proportionally added water to the flask and the consequent headspace vapor development that partially inhibited the volatilization of AITC. This effect was clearly visible from the flask glass wall that became opaque during experiments. Quantity of meal 0.1 g was chosen for the following trials because it was easier to handle than 0.05 g (<RSD%).

Table 2. AITC maximum yield determined after 70 min with different quantities of *B.carinata* DSM (g) and maintaining the meal to water ratio at 1:7.5 (g mL^{-1}). Mean values followed by the same letters are not statistically different ($P < 0.05$, Tukey's HSD test).

DSM:water (g:mL)	AITC $\mu\text{mol g}^{-1} \pm \text{SD}$	RSD %	AITC yield %
0.05 : 0.375	120.0 \pm 5.2	4.3	87.3 a
0.1 : 0.750	123.1 \pm 3.9	3.2	89.5 a
0.2 : 1.500	100.2 \pm 5.8	5.8	72.9 b

Different DSM to water ratios were then tested and 1:7.5 (g mL^{-1}) gave the best results with the maximum yield (**Table 3**). Minor quantity of water (1:5) were not sufficient to wet the meal homogeneously and data had a higher variability (>RSD%), while higher quantities (1:1 and 1:1.5) probably had the same above mentioned problem referring to humidity development.

Table 3. AITC maximum yield determined after 70 min with different amount of water and 0.1 g of *B.carinata* DSM. Mean value followed by the same letters are not statistically different ($P < 0.05$, Tukey's HSD test).

DSM:water (g mL ⁻¹)	AITC $\mu\text{moles g}^{-1} \pm \text{SD}$	RSD %	AITC yield %
0.1 : 0.50	99.5 \pm 8.1	8.1	72.4 b
0.1 : 0.75	123.1 \pm 3.9	3.2	89.5 a
0.1 : 1.00	106.7 \pm 8.0	7.5	77.6 b
0.1 : 1.50	112.3 \pm 3.3	2.9	81.7 ab

Maintaining the optimum found conditions, DSM quantity at 0.1 g L⁻¹ and DSM to water ratio at 1:7.5, a maximum AITC yield corresponding to 89.5%, was obtained after 60-70 min, that could be considered a relative short time for a quality control meal evaluation, especially if compared to classical GL analysis (**Figure 2**).

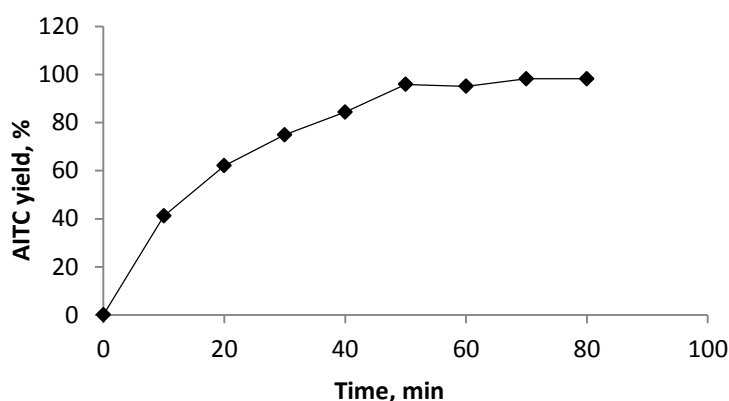


Figure 2. Allyl-isothiocyanate (AITC) kinetic release at optimized conditions of 0.1 g L⁻¹ DSM and DSM to water ratio at 1:7.5, 25 °C. AITC concentration was calculated from a previous determined calibration curve with pure AITC on the basis of the defatted seed meal containing sinigrin.

The remaining unrecovered 10.5 % of AITC could have been lost because bound to the DSM proteins or solubilized in the added water and not completely passed to the vapor phase, or for other unknown reasons. In order to investigate this aspect and to consider the DSM/water matrix effect a calibration curve was also constructed by adding pure AITC to a neutral, not ITC-producing, DSM (0.1 g), as sunflower, and water (0.75 mL). Calculation of AITC concentration utilizing the new calibration plot permitted to obtain a recovery of 98.3%, demonstrating that the presence of the meal and/or water prevented about 10% of AITC from going in the headspace.

Method applications

The method could be applied for the fast evaluation of the effect of different parameters on AITC realising properties, as meal particle size, GL and lipid content, MYR activity. It could also be employed to investigate the effect of factors that could affect MYR activity, as temperature, pH, ascorbic acid, magnesium chloride and so on (Dai and Lim, 2014; Sharma et al., 2012). In the present work the effect of meal oil content on AITC yield was confirmed by using the developed flask-model system. The kinetic release of *B.carinata* meals with three different oil content is shown in **Figure 3**.

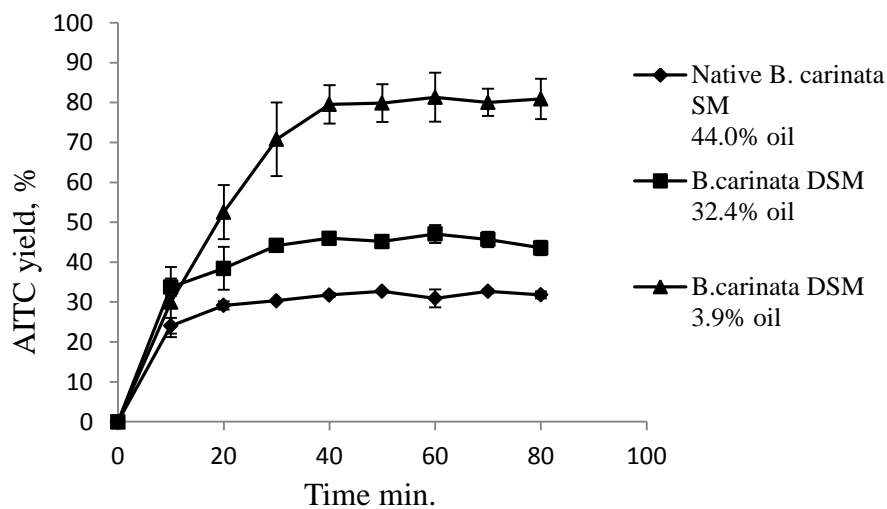


Figure 3. Allyl-isothiocyanate release from *B.carinata* meals with different oil content.

The AITC release capacity of the *B.carinata* meal was inversely correlated to the meal oil content (**Figure 4**) and native *B.carinata* with a 44% oil content had an AITC maximum yield as low as 32.7%.

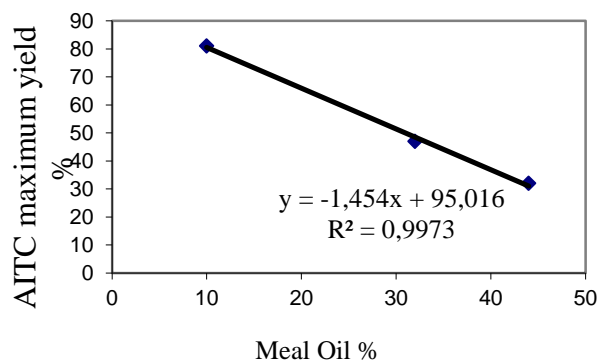


Figure 4. AITC releasing yield (%) as a function of *B.carinata* meal oil content (%).

The MYR activity of meals was also measured by pH-stat method (Franco et al., 2016) and it was similar to native and hexane extracted DSMs ($\sim 30 \text{ U g}^{-1} \text{ DSM}$), so it is not affected by the presence of oil. Therefore the different releasing behaviour could be ascribed to the retaining capacity of the oil on AITC as a lipophilic molecule. (Dai and Lim, 2014; Li et al., 2015).

Biofumigant material quality control

Deactivated *B.carinata* meal was defatted at the pressure plant and had a GL content of $95.4 \mu\text{moles g}^{-1}$ and a residual oil content of 10.7% (dry mass), higher than meals subjected to exhaustive oil extraction by hexane (3.9%). Kinetic plot relative to deactivated and different formulated DSMs are presented in **Figure 5**.

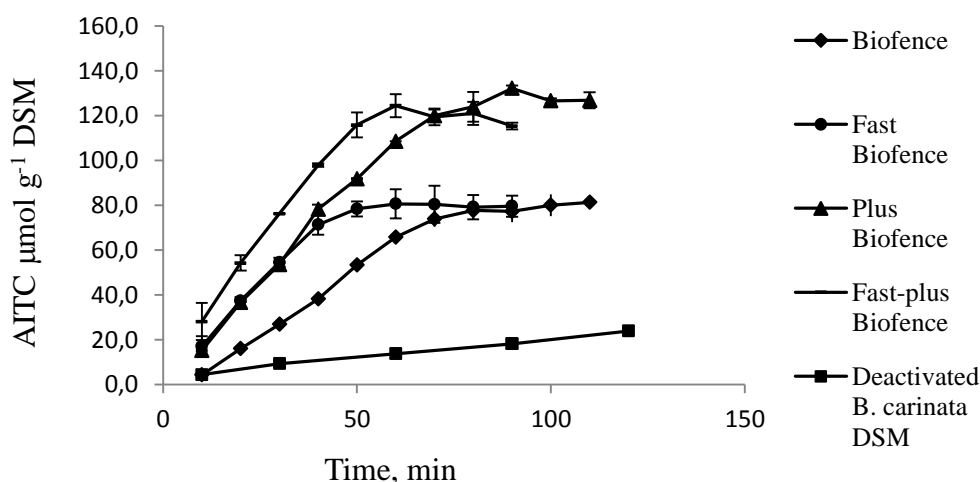


Figure 5. AITC releasing properties of biofumigating materials differently formulated.

It is clear from the graph that deactivated *B.carinata* DSM had a very slow and incomplete AITC release capacity and a maximum AITC concentration reached about $24 \mu\text{mol g}^{-1}$ after 120 min (25.2% yield). The releasing properties were restored in the formulate Biofence where AITC reached the concentration of $81 \mu\text{mol g}^{-1}$ (84.9% yield) in 110 min. Starting from this basic and commercial formulation, new experimental formulations with different behaviors were developed: Fast Biofence, instead, determined a faster AITC release which reached the same maximum AITC concentration and yield as with Biofence, but after only 50 min, while with Plus Biofence a higher amount of AITC ($132.2 \mu\text{moles g}^{-1}$, 92.4% yield) could be obtained in the same time (110 min), by increasing DSM GL content; in Fast-Plus Biofence both actions took place and

a higher amount of AITC ($124.5 \mu\text{moles g}^{-1}$, 87.0% yield) was released in a shorter time (60 min). Therefore the developed method permitted a rapid evaluation of formulate MYR-GL system changes and could be applied in biofumigant material quality control in order to verify, at a first look, its behavior. Furthermore it could also efficiently support the experimental development of new formulations, giving a rapid response on the modulation of AITC release from the meals.

3.2.4. Conclusions

The proposed analytical method is practical, fast and requires only a GC-FID instrument for measurements. It allows a rapid evaluation of the parameters which can affect meal AITC release, as MYR activity, GL and oil meal content. The method was also employed for the investigation and development of DSM formulations, but above all it is now routinely applied at laboratory level to every batch of commercial or experimental biofumigant material as a quality pathway for solid formulations: pellets or powder for mechanical or manual distribution, respectively applied as soil pre-plant treatment or fruit postharvest biofumigation (Section 3.3), but also DSMs as components of liquid formulations for ipogea and epigeal distribution, based on a vegetable oil in water emulsion at 1-2% (Lazzeri et al., 2011, 2013).

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3.3. The Biofumigation technique in fruit postharvest: evaluation of the Brassica derived allyl-isothiocyanate effect on the pathogen control and the fruit quality

The post-harvest system and the agro-food chain comprise multiple and interconnected technical and economic activities from the harvest to the household consumption phase, including transport, storing, processing, product evaluation and quality control, packaging and marketing (Grolleaud, 2017). Food losses can occur at any stage of the supply chain and represent the 5-20% of harvest product (in Italy 10%), but can easily reach the 50% in developing countries, due to technological limitation and/or difficult climatic conditions and inefficient infrastructure systems (Gustavsson et al., 2011). The consequent economic loss is cumulative throughout the chain and becomes more important when it involves the last stages of the product processing/distribution. A more efficient and sustainable management of the entire food chain, a major awareness among industries and consumers, is necessary to reduce food losses and increase the quality and quantity of food supply in a sustainable approach, also in view of population increase (Gustavsson et al., 2011, El-Ramady et al., 2015).

In this context, storage can be considered the postharvest phase where different management strategies could be applied in a more controlled way than pre-harvest strategies in the field, even if a holistic approach and an integrated management program is often necessary to minimize postharvest losses and to substitute phytochemical uses (Romanazzi et al., 2016a). Food losses can be quantitative (physical reduction in weight and volume) but moreover qualitative referring to the edibility and food safety and nutritive/nutraceutical value in term of health promoting phytochemical content. In addition, the consumption of fresh fruit and vegetables, in particular, is strongly recommended as they are products rich of water, fibres, minerals, and are an important source of vitamin C, A, potassium, flavonoids and other bioactive molecules involved in antioxidant mechanisms and in prevention of cancer and cardiovascular diseases (Yahia, 2017). Postharvest fruit losses can be caused by physiological disorders, linked to senescence or physio-pathologies as scald or chilling injuries. Nevertheless the main cause of losses is the microbiological decay, attributed to pathogen fungi of the genera of *Penicillium*, *Botrytis*, *Monilinia*, *Rhizopus*, *Alternaria*, *Fusarium* etc (Mari et al., 2014).

Postharvest handling practice and its success depends on the type of product: perishable horticultural crops, which can be stored for few days, as strawberries, and low-perishable goods, as pear, apple and kiwifruit. Temperature (usually kept between 0 and 10 °C), humidity and atmosphere gas composition are some of the main physical parameters which influence product maintenance (Romanazzi et al., 2016a). Until now synthetic fungicides have been the first line of

defence in conventional agriculture even though the European and local legislation regarding quantity and quality of permitted molecules and maximum residue limits, have been more and more strictly regulated, aiming to a higher human and environment safety. In postharvest phase, the list of the allowed phytochemicals depends on the country and is limited to a few active ingredients for most commodities, while for other, chemical treatments are completely banned. EU stated rules for the sustainable use of pesticides to reduce their risks and the impact on people health and environment (Directive 2009/128/EC). In Italy the use of postharvest phytochemical is not allowed for stone fruit while pre-harvest fungicide applications are performed, even if chemicals used for pome and kiwi fruit (es thiabendazole) are frequently ineffective (Mari et al., 2014). The research of alternative solutions to fungicides is in fact also driven by the insurgence of pathogen resistance, consequence of the chemical systemic application, and by an increasing attention on food safety. Consumers more and more often request foods with lower residues, safe, and environmentally and economically sustainable. The EU encouraged the scientific research for an integrated pest and disease management to reduce the use of pesticides in the horticultural sector by including, in the Horizon 2020 program framework part ‘Food security, sustainable agriculture and forestry, marine and maritime and inland water research and the bioeconomy’, the so called ‘Sustainable food security – resilient and resource efficient value chain’ and in particular in the topic of ‘Innovations in plant protection (SFS-17-2017⁹)’.

Among alternative strategies the most promising are those based on the use of microbial biocontrol agents, which act through different modes of action including antibiotic or lytic enzyme production and competition for nutrient and space (Janisiewicz and Korsten, 2002; Liu et al., 2013); plant elicitors (chitosan) which have the indirect effect of plant defense system induction (Romanazzi et al., 2016b); disinfecting agents; physical treatments (heating, irradiation, ozone, waxing) and the use of plant secondary metabolites with antimicrobial activity (Mari et al., 2011; Hintz et al., 2015). Glucosinolate (GL)-derived isothiocyanates (ITCs) belong to the latter category and are known for their strong biological activity on fungal pathogens, even of postharvest relevance, and also against several food-borne pathogens dangerous for human health (Delaquis and Mazza, 1995; Wilson et al., 2013; Dufour et al., 2015).

Some ITCs are characterized by high volatility and have been exploited for gaseous treatments of fruit and vegetables, as in the ‘biofumigation’ technique, to increase storage time, giving successful results in *in vitro* and *in vivo* studies (Troncoso-Rojas et al., 2005; Chanjirakul et al., 2007; Troncoso-Rojas et al., 2009; Wang et al., 2010; Manyes et al., 2015; Sotelo et al., 2015;

⁹ <https://ec.europa.eu/research/participants/portal/desktop/en/opportunities/h2020/topics/sfs-17-2017.html>

Chen et al., 2015). In previous studies, a treatment based on an atmosphere enriched in specific ITCs was found to be active against *Penicillium expansum* on pears (Mari et al., 2002) and *Monilinia laxa* on peaches and nectarines (Mari et al., 2008), while on *B. cinerea* there are only few data from *in vitro* and *in vivo* trials (Mari et al., 1993, 1996). Among ITCs tested in the cited works, allyl-isothiocyanate (AITC), produced from the GL sinigrin (2-propenyl-GL), seems to be one of the most active, for biological activity and favourable vapour pressure (sections 3.1 and 3.2). Furthermore, AITC is a molecule ‘generally recognized as safe’ (GRAS) by the Food and Drug Administration (FDA) in the United States since 2006 (Manyes et al., 2015) and, if derived from plant sources, is authorized as a natural food preservative in Japan. The European Food Safety Authority (EFSA, Parma, Italy) evaluated its safety as a food additive with interesting results (EFSA 2010). Recently, several studies have been directed towards the use of AITC incorporated in antimicrobial food packaging systems as modified atmosphere packaging (Issiki et al., 1992; Delaquis and Mazza, 1995; Nielsen and Ros, 2000; Winther and Nielsen 2006) or active packaging with AITC-films (Hendrix et al., 2012; Seo et al., 2012; Dias et al., 2013) in order to increase significantly the product shelf life.

AITC demonstrated its antimicrobial activity *in vivo* either applied as a synthetic compound or as GL-derived ITC produced *in situ* from renewable sources as cabbage leaves (Troncoso-Rojas et al., 2005) or *Brassica carinata* defatted seed meal (DSM) (Mari et al., 2008), thus making the process economically and environmentally sustainable. In order to investigate the AITC effect *in vivo* on a small pre-pilot scale, a system was developed at the CREA-CIN laboratories, consisting in a cabinet of 0.1 m³, connected to an external reactor where the production of AITC from GL-MYR system of *brassica* DSMs was achieved by a simple water addition. The obtained rich AITC vapor phase was then pumped inside the cabinet with a continuous flow of around 250 L h⁻¹ from and to the external reactor in a closed circuit (Mari et al., 2008). Up to 4 trays of fruit could be treated in one trial. The AITC concentration was continuously monitored by gas-chromatographic-flame ionization detector (GC-FID) analysis throughout the atmosphere sampling in order to maintain it at the established value (**Figure 1**).



Figure 1. Cabinet (0.1 m³) for postharvest fruit treatment with allyl-isothiocyanate (AITC) produced from *brassica* defatted seed meals (DSMs) in an external reactor and pumped inside the treatment cell (on the left). AITC atmosphere concentration GC-FID continuous monitoring system (on the right).

The system was also preliminarily verified at a pilot scale in refrigerated treatment/storage cells (30 m³) with interesting results on pear and peaches even if the technology needed further set-up studies (Bernardi et al., 2005).

While the antimicrobial activity of AITC vapours is well known, the effects of the molecule on fruit quality attributes, related to appearance, flavour and nutrient properties are less investigated even if of fundamental relevance for the final product appeal to the consumer and marketability. The molecule is highly reactive and a low concentration is needed for pathogen control while at the same time, its volatility determines low residue content in treated goods (Isshiki et al., 1992; Mari et al., 2002). Anyway further studies are necessary to know if AITC adducts are formed in the fruit, for instance by their known reaction with protein thiols. The effect of the molecule on fruit general appearance had no detrimental effect in netted melon and in bel pepper as observed by Troncoso-Rojas (2005, 2009) after ITC treatment. Moreover the product organoleptic quality should also be taken in consideration and some studies showed positive results on the effort of neutralizing the AITC pungent smell, which badly characterises it, by appropriate formulations (Ko et al., 2012; Wu et al., 2015).

Regarding the AITC effect on plant and the cellular and molecular mechanisms of action, few investigation have been performed until now. As mentioned before, AITC is a reactive molecule binding thiols, such as cysteine or glutathione (GSH), thus modifying protein structure and

function. Khokon et al. (2011) observed that AITC induced stomata closure in *Arabidopsis thaliana* via production of reactive oxygen species (ROS) and nitric oxide, probably with the function of preventing water loss and enhancing plant defence against microorganisms. At higher doses it also caused oxidative stress and ROS production in *Arabidopsis* seedlings, while expression of glutathione S-transferase (GST)-encoding genes and reversible GSH depletion was observed at low doses, as a potential detoxifying mechanism (Øverby et al., 2015). Besides, some works speculate on the AITC function as ethylene analogue or inhibitor based on their structural similarity, but this action has not been fully investigated yet (Nagata, 1996).

At present, from the standpoint of fruit crops, postharvest AITC biofumigation has been successfully applied to limit fungal decay in different kinds of berries (e.g. raspberries, strawberries, blackberries, blueberries), apparently due to its direct effect on fungi and pro-oxidant activities. Wang et al. (2010) suggested that the observed increased ROS production in blueberries could be a possible defence mechanism induced in AITC-treated fruits. Nevertheless, the treatment was reported to have a detrimental effect on the antioxidant phytochemical content of these fruits even though a global fruit quality improvement was observed (Chanjirakul et al., 2006; Chen et al., 2015).

In the sections 3.3.1. and 3.3.2. the results of the study of the *in vivo* effect of AITC vapour, produced from *brassica* DSMs with the small pre-pilot system described above, on the fungal pathogen *Botrytis cinerea* and on the fruit quality and nutraceutical properties are presented on two different host fruit, strawberry and kiwifruit.

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3.3.1. Control of postharvest grey mould (*Botrytis cinerea* Per.: Fr.) on strawberries by glucosinolate-derived allyl-isothiocyanate treatments.

3.3.1.1. Introduction

Strawberries (*Fragaria x ananassa* Duch.) are fruits characterized by unique taste and flavour, highly appreciated by consumers, but also by healthy properties as they are rich in natural antioxidants as polyphenols and anthocyanin, vitamins and amino acids. They are a good sources of vitamin C, L-Ascorbic acid (around 40-70 mg/100 g strawberries, FW), which is an essential nutrient in human diet, thanks to its important antioxidant and metabolic functions, protecting from carcinogenesis and cardiovascular diseases and stimulating the immune system (Sapeia and Hwaa, 2014; Du et al., 2009). Anyway vitamin C and other nutrient content strongly depends on fruit ripening stage, storage conditions and cultivar. Gray mould caused by *Botrytis cinerea* Per.: Fr. is one of the main disease of strawberries occurring both in the field and after harvest (Droby and Lichter, 2007). Losses can be severe, reaching up to the 25% in the main harvest and the 37% in the second. However the main losses happen during the postharvest phase, with a maximum incidence of 89% in the second harvests (Ceredi et al., 2009). Chemical control is essential to prevent pre and post-harvest fruit decay; in conventional agriculture anilinopyrimidine, cyprodinil, phenylpyrrole and fludioxonil had been widely applied for grey mould control for more than 10 years. Since all these active ingredients are site-specific inhibitors, the appearance of resistance has to be considered with great attention (Fernández-Ortuno, et al., 2013), suggesting the need of an alternative to these products, overall in organic agriculture where treatments with synthetic fungicides are not admitted. Several non-conventional methods have been recently investigated with encouraging results, among which the use of chitosan (Romanazzi et al., 2013), of biocontrol agents (Huang et al., 2012) and of physical methods, as pre-storage hypobaric treatment (Hashmi et al., 2013) or hot water treatment (Villa-Rojas et al., 2011). Treatments with chitosan-based edible coatings also showed to maintain better strawberry quality and treated fruits had higher contents of antioxidants (phenolics, anthocyanins, flavonoids), ascorbic acid, glutathione and antioxidant enzyme activity, than untreated fruits, thus reinforcing the resistance against fungal invasion and extending fruit shelf-life (Wang and Gao, 2014). Natural substances with biological activity could be an interesting option that has been less investigated and few experiences are reported on the efficacy of natural compounds against *B. cinerea* on strawberries. Archbold et al. (1997) tested some natural volatile compounds for their antifungal activity against pathogens on artificially inoculated strawberry fruits. Wang et al. (2007) showed the efficacies of natural essential oils derived from plants (thymol, eugenol, menthol) in inhibiting decay and increase free radical scavenging capacity and

anti-proliferative activity in strawberry, thus extending fruit shelf life. Chanjirakul et al. (2007) evaluated the effect of methyl jasmonate, allyl isothiocyanate (AITC), essential oil of *Melaleuca alternifolia* and ethanol on strawberries and blackberries, and found that AITC gave the best results in terms of reduction of decay severity (not specified of which nature), with a less important effect on the overall free-radical scavenging fruit capacity.

Since strawberries, after harvest, are particularly perishable fruits, highly susceptible to rapid deterioration and spoilage, but also to *B. cinerea* infections, the aim of this work was overall to define a strawberry postharvest treatment based on the natural antimicrobial compound AITC that in preliminary experiences showed to be able to reduce decay and to increase the fruit shelf-life. In particular, the present study addressed the following objectives: (i) to determine the EC₅₀ and EC₉₅ of synthetic AITC on conidia germination and mycelial growth of *B. cinerea*; (ii) to evaluate *in vivo* the activity of synthetic or GL-derived AITC against *B. cinerea* on two naturally infected strawberry cultivars; (iii) to assess AITC residues on strawberry fruit after treatment; (iv) to investigate the influence of AITC treatment on the nutraceutical and antioxidant characteristics of strawberry as fruit antiradical capacity, and anthocyanin, polyphenol, ascorbic acid and glutathione content.

3.3.1.2. Material and Methods

Pathogen

B. cinerea strain was isolated from strawberries showing typical grey mould infection. The pathogen was grown on oat meal agar (60 g of oatmeal, 10 g of sodium nitrate, 30 g of saccharose and 12 g of agar per 1000 mL of distilled water), and the culture was incubated at 25°C under UV (350-420 nm) light for 12 h daily for 10 days. The isolate was maintained on malt extract agar (MEA) slant at 4°C. For the *in vitro* trials, a conidial suspension was prepared by washing the colonies of pathogen with sterile distilled water containing 0.05% (v/v) Tween 80, and the concentration of suspension was quantified by a hemacytometer and diluted to the final concentration of 10³ conidia mL⁻¹.

Isothiocyanate

The chemical AITC was obtained from Aldrich Chemical (> 98%, Milwaukee, WI, USA). GL-derived AITC was released from *B. carinata* defatted seed meal (DSM), produced by mechanical oil extraction and appropriately formulated by a patented procedure able to modulate AITC release in time after watering (Lazzeri et al., 2010; section 3.2.). Meal was characterized for its moisture content, residual oil and GL content as described in De Nicola et al. (2013).

Effects of AITC on conidia germination and mycelium growth in in vitro trials

The inhibition of conidia germination was tested by spreading 100 μL of pathogen conidia suspension (10^3 conidia mL^{-1}) on Petri dishes containing 20 mL of MEA. Mycelium growth inhibition was evaluated by placing a plug (6 mm diameter) from an actively growing culture in the centre of a MEA plate. In each case, different aliquots of pure AITC (0.08, 0.16, 0.32, and 0.64 μL x Petri dishes) were placed, using a microsyringe, on a 90 mm diameter paper filter (Whatman N $^{\circ}$. 1), positioned inside the cover. The dishes were quickly closed, sealed by Parafilm and incubated at 20°C. The dishes were opened to evaluate AITC fungicidal activity of after 2 days for conidia germination and after 3 days for mycelium growth (first evaluation); the paper filter was subsequently removed, allowing the fungus to grow for the next 2 and 3 days respectively (second evaluation). Petri dishes inoculated with the pathogen, but treated with distilled water were used as a control. Five replications were used for each treatment (different AITC concentrations) and the experiment was repeated three times. Conidia germination was determined as reported by Mari et al. (2008). Mycelium growth was gauged with a centimetre ruler. EC_{50} and EC_{95} were calculated as the concentration that inhibits conidia germination or mycelium growth by 50% and 95 % respectively, compared to the control.

Fruit

Three strawberry cultivars were used: ‘Tecla’ a spring-bearing cultivar; ‘Monterey’ a day-neutral cultivar and ‘Clery’ used for antioxidant and phytochemical content evaluations. All of them were cultivated in experimental fields located in the Romagna region (Northern Italy) under organic management; ‘Tecla’ and ‘Clery’ strawberries were harvested in the last decade of May and ‘Monterey’ strawberries in the first decade of October. Fruit free from evident wounds and rot and homogeneous in maturity and size were selected and treated on the same day as harvest.

Treatment

Selected strawberries were distributed in baskets (almost 250 g of fruit each) on two trays placed in a 0.1 m^3 sealed cabinet and treated by AITC vapours as described in Mari et al. (2008) (section 3.1). Preliminary trials were conducted on ‘Tecla’ strawberries by injecting aliquots of pure synthetic AITC directly in the cabinet, at different times during treatment, to maintain a constant atmosphere concentration. In order to determine the time of exposure and AITC concentration able to contain the pathogen and at the same time to avoid phytotoxic effect on fruit, two different concentrations of commercial AITC (0.1, 0.2 mg L^{-1}) and different treatment times (2, 3, 4 and 6 hours) were tested. Synthetic AITC was then substituted with AITC released from DSM by

endogenous GL (sinigrin) - MYR system. In this latter case, 10 g of DSM were placed in an external 500 mL flask with 30 mL of water. The gas phase, AITC-enriched, was pumped into the cabinet at 250 L h⁻¹. AITC concentration was maintained constant by flow regulation of the air pump and was determined by atmosphere sampling at regular intervals followed by gas chromatography-flame ionization detector (GC-FID) analysis as described below. In order to confirm the activity of GL-derived AITC also on a day-neutral cultivar, even a trial on 'Monterey' strawberries was conducted, using the optimum treatment conditions previously defined as 0.1 mg L⁻¹ of AITC for 4 hours. In all experiments, control fruits were represented by untreated fruits placed in the same conditions. After treatment, fruits were stored for 2 days at 0°C and then at 20°C for another 3 days (shelf-life). Finally, the percentage of infected fruit was recorded. The sample unit was represented by 10 replicates (baskets) of 15 fruit each, unless differently specified. Effective index was calculated as control incidence – treated incidence/control incidence x 100.

Headspace gas chromatographic analysis

The atmosphere in the cabinet was monitored by an Agilent 7820AC GC system equipped with a Varian HP-5 column (30 m length, 0.32 mm inner diameter, 0.25 µm film thickness) and FID detector. Instrument settings were as follows: injector and detector temperature were set at 200°C and 300°C respectively; the oven program started at 60°C and raised to 120°C at a rate of 10 °C min⁻¹; the carrier gas (He) flow rate was 1 mL min⁻¹ and the splitless injection mode was selected. The AITC amount was determined on the basis of a previously defined calibration curve using a pure AITC standard.

AITC residue analysis in fruits

AITC residue analysis were carried out on 'Tecla' strawberries treated by GL-derived AITC, 0.1 mg L⁻¹, for 4 and 6 h in order to investigate the influence of treatment duration on residue level. Residue determination was performed on fruit one hour after the treatment and at the end of fruit storage (2 days at 0°C and 3 days at 20°C), corresponding to consumption time. The solid phase micro-extraction, SPME, technique coupled with GC-FID analysis was used. Treated fruits randomly chosen from the trays (two per basket) were cut into small pieces and samples of 5 g were introduced in a 20 mL vial. Sample pre-incubation, extraction and injection on GC were performed through an automatic auto-sampler CombiPAL (CTC Analytics) under the following conditions: 10 min pre-incubation in the heated agitator block at the constant temperature of 80°C and agitation speed of 250 rpm; insertion of the SPME needle in the sample vial and exposure of the fibre for 10 min extraction; insertion of SPME needle in the injector port and exposure of the fibre for 2 min at 260°C, for compound thermal desorption. The extraction fibre used was coated with 75 µm

Carboxen-polydimethylsiloxane film (CAR-PDMS) (Supelco, Bellefonte, USA). GC analysis was carried out on a Varian GL-3800 equipped with a J&W DB23 column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness) and FID detector. The analytical conditions were: injector temperature 260°C; detector temperature 280°C; flow 1 mL min⁻¹ of the carrier gas (nitrogen); column oven temperature initially maintained at 50°C for 6 min, followed by a gradient to 220°C, with a rate of 40 °C/min, finally held for 2 min. The splitless mode was used for injection. Blank samples of untreated fruit were analysed following the same protocol. AITC quantification was performed plotting a calibration curve by spiking blank samples of non-treated fruit with 10 μL AITC standard solutions in hexane, at different concentrations, in order to minimize the matrix effect of fruits on AITC determination. Measurements were replicated 8 times for treated fruits and 5 times for calibration samples.

Antiradical capacity and phytochemical content determination

For the evaluation of the effect of AITC treatment on the antioxidant and nutraceutical parameters of fruit, 'Clery' strawberries were treated as described above (0.1 mL L⁻¹ for 4h), then stored at 20°C for two days. At the end of storage (d2), 5 pools of 10 treated and untreated fruits (one-fourth of fruit), with no physical injury, diseases, or incidences of pests, were selected and frozen in liquid nitrogen, grinded by a Waring Commercial laboratory blender (Waring products division Torrington, CT, USA) and stored at -80°C.

Antiradical capacity, anthocyanin and total phenolic content. 50 mg of frozen strawberries (d2) were extracted with 1 mL of solution of methanol:water (80:20 v/v) acidified with 0.1% HCl (v/v), by using ultrasound-assisted extraction (20 min). For anthocyanin determination the same method was used except for solvent methanol:water (80:20 v/v) and 0.15 M HCl. After centrifugation (30,500 g, 10 min, 4 °C) supernatants were collected and immediately analysed. Total phenolic content was determined following the spectrophotometric Folin-Ciocalteu method (765 nm), using gallic acid (GA) as standard (Weitz et al., 2010). Six serial dilution of samples and standards were performed and the slope ratio of the two obtained calibration curves (sample/standard) were calculated. Blank sample with extraction solvents without samples or fruit extracts without colouring reagents were considered. Results were expressed as mg of GA equivalent (mg GAE) per g of fresh material. Anthocyanin content was determined by following the Gould et al., (2000) spectrophotometric method by using the cyanidin-3-*O*-glucoside (Kuromanin) chloride external standard calibration curve. Samples absorbance was recorded at 530 and 653 nm. Antiradical capacity was assessed by measuring the ability of the extracts to scavenge synthetic radicals [i.e. 2,2-diphenyl-1-picryl-hydrazyl-hydrate, DPPH• and 2,2'-azinobis-(3-

ethylbenzothiazoline-6-sulfonic acid), ABTS^{•+}]. Both DPPH[•] and ABTS^{•+} quenching capacities were estimated spectrophotometrically, and expressed in terms of EC₅₀ (Carbone and Mencarelli, 2015). Reference antioxidant standard trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and BHA (butylated hydroxyanisole) were used for assay calibration and results were also expressed as μmol of trolox equivalent (TE) or of BHA equivalent (BHA-E) per g of fresh sample.

All spectrophotometric assays were performed by using an Infinite M200 PRO microplate reader and measurements were replicated two times for each pool.

Ascorbic acid content. Sampling was performed as described above, at different times: at harvest (H), soon after treatment (d0) and after one (d1) and two days (d2) of storage at 20°C. Ascorbic acid was extracted three times from frozen samples with 20mM NaH₂PO₄, containing 1mM ethylenediaminetetraacetic acid, EDTA, pH 2.1. (1:4 w/v), by using a TissueLyser (Retsch GmbH, Haan, Germany). After centrifugation (31,500 g, 20 min, 4 °C) supernatant were filtered and used for analysis. Reduced ascorbic acid (AsA) content was determined by HPLC analysis by using a Hewlett-Packard chromatograph 1100 equipped with a diode array detector, a ChromSep HPLC column SS (250 × 3.0 mm) and a ChromSep guard column Intersil 5 ODS-3 (Varian). AsA was detected at 245 nm and the mobile phase A and B were 20 mM KH₂PO₄, pH 2.5 and 60% methanol - 40% acetonitrile, respectively (Agilent application note 5990-8720EN). AsA standard (Sigma Aldrich) was used for external calibration. Dehydroascorbic acid (DHA) was reduced to AsA with tris(2-carboxyethyl)phosphine (TCEP) reducing agent, by incubating 200 μL of diluted extract and 50 μL of 50 mM TCEP (pH 2.1) for 60 min, at 4°C, in the dark. DHA was calculated as the difference between the total ascorbic acid (reduced plus oxidized, AsA + DHA) and AsA. AsA and DHA contents were expressed as mg/100g FW. The redox status of ascorbate pool was calculated as $\text{AsA}/(\text{AsA}+\text{DHA}) \%$.

Glutathione content. Frozen ground strawberry samples collected from fruit at d0, d1 and d2 were extracted with cold 5% 5-sulfosalicylic acid (SSA), 1:10 w/v, at 4°C by using a TissueLyser and subsequently centrifuged at 31,500 g for 15 min at 4°C. Total glutathione (reduced + oxidized glutathione, GSH + GSSG) spectrophotometric analysis was performed by using the GSH assay kit (Sigma-Aldrich), with GSH reductase, 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) and NADPH, according to the provided protocol on an Infinite M200 PRO microplate reader. GSSG was determined by the same method in the presence of GSH masking agent 1-methyl-2-vinylpyridinium triflate (M2PV) (Shaik and Mehvar, 2006). Results are expressed as nmol g^{-1} FW. GSH was estimated as the difference between total and oxidized form and GSH redox state calculated as the percentage of GSH over total: $\text{GSH}/(\text{GSH}+\text{GSSG}) \times 100$.

Statistical analysis

EC₅₀ and EC₉₅ values were calculated using the probit analysis applied to the percentage of conidia germination and mycelial growth inhibition (Lesaffre and Molenberghs, 1991). All other data were subjected to one-way analysis of variance (ANOVA) using Statistica 8.0 software (Statsoft, USA). Data were reported as mean values \pm standard error (SE), separation of means was performed using the least significance difference (LSD) test, at $P < 0.05$, unless differently specified. All experiments were carried out in a completely randomized design.

3.3.1.3 Results

Effects of AITC treatment on conidia germination and mycelium growth

AITC vapours were tested on conidia germination and mycelial growth of *B. cinerea* in *in vitro* trials. The mycelium appeared to be less sensitive to AITC than conidia, with an EC₅₀ value for mycelial growth (1.35 mg L⁻¹) almost double the EC₅₀ for conidial germination (0.62 mg L⁻¹) (**Table 1**). In addition, the AITC showed a fungistatic effect against the pathogen since at the second evaluation, two or three days after Petri dish opening and after removal of the paper filter, the values of EC₅₀ for both parameters increased by almost 30%.

Table 1. EC₅₀ and EC₉₅ values (mg L⁻¹) of synthetic allyl-isothiocyanate vapours on conidia germination and mycelial growth of a *Botrytis cinerea* isolate.

Biological parameters	1 st evaluation ^a		2 nd evaluation ^a	
	EC ₅₀	EC ₉₅	EC ₅₀	EC ₉₅
Conidial germination	0.62	1.04	0.86	1.54
Mycelial growth	1.35	2.83	1.97	3.94

^aThe evaluations were carried out after 2 and 3 days for conidial germination and mycelial growth respectively (1st) and 4 and 6 days (2nd) after Parafilm and paper filter removal.

AITC fruit treatment

Some preliminary experiments carried out with synthetic AITC showed that the exposure to AITC vapours at a concentration of 0.1 mg L⁻¹ for 4 hours determined no phytotoxic effect on strawberry fruit and a significant reduction of infection ($P < 0.018$) over 45%, if compared to the control. On the contrary, treatments with AITC concentration of 0.2 mg L⁻¹ for 2 or 3 hours or 0.1 mg L⁻¹ for 6 hours did not improve efficacy of grey mould infection control compared to results

obtained with 0.1 mg L⁻¹ for 4 hours (data not shown). Therefore, treatment conditions were established at AITC 0.1 mg L⁻¹ and 4 hours for all subsequent trials. GL-derived AITC was produced *in situ* from formulated DSM with defined characteristic as 6% moisture content, 10.9% residual oil and GL content of 80.92 µmol g⁻¹ (on dry matter). In the case where strawberries with a high level of infection were considered, after 2 days of storage at 0°C and another 3 days at 20°C, untreated fruit showed an incidence of grey mould that varied from 44.7 to 40.1 (**Table 2**). AITC treatment significantly reduced the losses caused by the pathogen, with a reduction of 47.4% (synthetic AITC) and 48.9% (GL-derived AITC). In the second case, the trials were performed with a lower disease pressure; the control fruit showed an incidence of grey mould of 13.0% and 8.9% in the two sets of trials. AITC treatment reduced *B. cinerea* infections better than in the experiments with high disease pressure: the pathogen reduction was 91.5% for synthetic AITC and 86.5% for GL-derived AITC. In all trials, no differences were observed in the efficacy associated with different AITC sources.

Table 2. Effect of allyl-isothiocyanate (AITC) vapours from synthetic and GL-derived origin on *Botrytis cinerea* (%) in ‘Tecla’ strawberries naturally infected (high and low disease levels), after 2 days at 0°C plus 3 days at 20°C. AITC treatment was performed at 0.1 mg L⁻¹ for 4 hours. Values for each trial correspond to the mean of 15 fruit per ten replicates. Mean values ± SE within the same treatment, followed by different letters are significantly different according to the LSD test (*P*<0.05)

Disease Level	AITC source	Control	AITC	Effective Index ^a (%)
High	Synthetic	44.7 ± 7.27 a	23.5 ± 3.67 b	47.4
	GL-derived	40.1 ± 2.95 a	20.5 ± 4.46 b	48.9
Low	Synthetic	13.0 ± 4.19 a	1.1 ± 0.76 b	91.5
	GL-derived	8.9 ± 2.17 a	1.2 ± 0.78 b	86.5

^aEffective index = (control incidence – treated incidence) / (control incidence) x 100

The incidence of disease in untreated day-neutral strawberry cultivar (‘Monterey’) was high (> 54%) however, the GL-derived AITC treatment confirmed its efficacy, significantly reducing the grey mould by more than 38% (**Table 3**).

Table 3. Effect of allyl-isothiocyanate (AITC) vapours from GL-derived origin on *Botrytis cinerea* (%) in day-neutral ‘Monterey’ strawberries naturally infected, after 6 day at 0°C plus 2 days at 20°C. AITC treatment was performed at 0.1 mg L⁻¹ for 4 hours. Values for each trial correspond to the mean of 15 fruit per ten replicates. Mean values ± SE within the same trial, followed by different letters are significantly different according to the LSD test (P<0.05).

Trials	Control	AITC	Effective Index ^a (%)
1st	54.5 + 4.77a	23.4 ± 4.31b	57.1
2nd	59.2 + 3.72a ^b	28.9 ± 4.55b	51.2
3th	59.2 + 3.72a ^b	36.2 ± 5.44b	38.9

^aEffective index= (control incidence–treated incidence)/(control incidence) x 100

^bA control was used for two different trials, performed in the same day.

AITC residues in treated fruit

Fruit AITC residue quantification, performed by the SPME GC-FID technique, was achieved by using the designed calibration curve which showed an excellent linearity under the investigated concentration range ($R^2 \sim 0.99997$) (**Figure 1**).

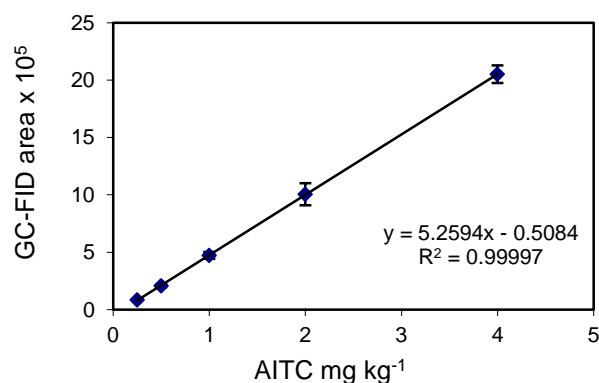


Figure 1. Calibration plot of pure allyl-isothiocyanate (AITC) for fruit residue determination: AITC gas chromatography-flame ionization detector (GC-FID) area vs AITC concentration in spiked blank samples of non-treated fruit (mg kg⁻¹). Samples for each concentration were replicated 5 times and mean values ± SE are reported.

Analysis on fruit treated with GL-derived AITC showed a residual presence of AITC that did not significantly differ at the two sampling times, 1 hour and 3 days of storage at 0°C plus 4 days of

shelf-life after treatment. AITC residue was in fact 0.93 and 0.91 mg kg⁻¹ of fruit in both cases. When the treatment duration was increased from 4 to 6 hours, a higher AITC residue in fruit was observed: 2.36 and 1.53 mg kg⁻¹, 1 hour and 7 days after treatment, respectively (**Figure 2**).

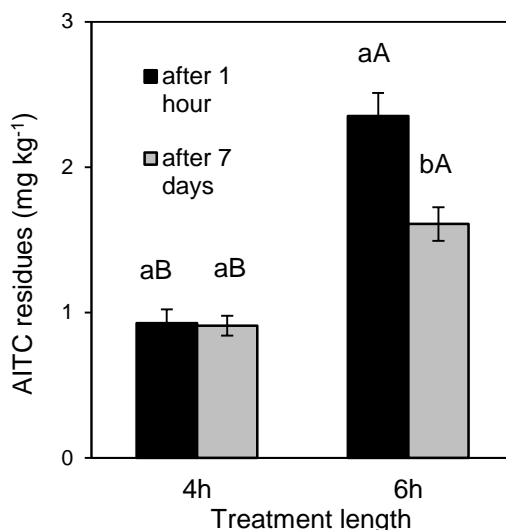


Figure 2. Influence of glucosinolate-derived AITC treatment duration on AITC residues (mg kg⁻¹) in naturally infected ‘Tecla’ strawberries. Residue analyses were performed 1 hr after treatment (black column) and at consumption time, 7 days of storage (grey column). Treatment dose was maintained at 0.1 mg L⁻¹, for 4 and 6 hours. Values for each trial correspond to the mean of 8 samples per treatment. Mean values \pm SE followed by different letters (lower-case-treatment length; capital-case-hr after treatment) are significantly different according to the Least Significance Difference (LSD) test ($P < 0.015$); no AITC was detected in control fruit under the Limit of Detection (0.019 mg kg⁻¹).

In this case, AITC detected one hour after treatment was higher but, as a consequence of the high volatility of the molecule, rapidly decreased to the stable value that was recorded one week later. At the same time, if referred to the activity of GL-derived AITC against grey mould, a longer treatment of 6 hours did not improve the control of the pathogen; indeed, the effective index, rated as 85.8% (with low level of infection, equal to 15.7% in untreated fruit) was similar to those found for treatment of 4 hours discussed above (**Table 2**). No AITC was detected in control fruit (Limit of detection 0.019 mg kg⁻¹) (Sanagi et al., 2009).

Effect of AITC on antioxidant and nutraceutical properties of strawberries

The main nutraceutical and antioxidant parameters in treated and untreated strawberries were evaluated.

Antioxidant capacity, total phenolic and anthocyanin content. Antiradical capacity was expressed as EC₅₀ or as standard (trolox and BHA) equivalents and in all cases resulted lower in treated samples respect to control, even if the differences were not significant. Total phenolic and anthocyanin content seemed also not to be significantly affected by treatment (**Table 4**).

Table 4 - Effect of GL-derived allyl-isothiocyanate (AITC) vapours treatment on nutraceutical parameters (means ± SE) in frozen ‘Clery’ strawberries after AITC treatment and two days of storage at 20 °C. AITC treatment was performed at 0.1 mg L⁻¹ for 4 hours. Results are expressed on fresh weight (FW). Antioxidant capacity is expressed as ABTS•+ or DPPH• EC₅₀ (mg)^a or μmoles g⁻¹ trolox equivalent (TE) or μmoles g⁻¹ butylated hydroxyanisole BHA equivalent (BHAE); TPC (mg gallic acid equivalents GAE g⁻¹); anthocyanin (mg Cya-3-glu g⁻¹). Mean values ± SE (LSD test, P<0.05).

		EC₅₀ ABTS•+	EC₅₀ DPPH•	TPC	Anthocyanin
Control	IC ₅₀	0.163 ± 0.031	0.065 ± 0.011		
	TE	12.24 ± 2.52	9.02 ± 1.05	2.04 ± 0.62	1.13 ± 0.12
	BHAE	12.25 ± 2.52	11.09 ± 1.29		
AITC	IC ₅₀	0.198 ± 0.018	0.076 ± 0.008		
	TE	10.18 ± 0.98	8.51 ± 1.06	2.18 ± 0.39	0.94 ± 0.11
	BHAE	10.29 ± 0.98	10.46 ± 1.30		

^aEC₅₀ = mg of strawberry required to obtain 50 % DPPH• or ABTS•+ scavenging

Ascorbic acid content. Reduced and oxidised form of ascorbic acid (AsA and DHA) and the redox status were determined at the time of harvest, soon after AITC treatment and after storage at 20°C, in AITC treated and untreated samples. ‘Clery’ strawberries showed to have a good ascorbic acid content at the harvest, which was mostly preserved during the storage in treated and untreated samples. Results showed, in fact, no significant differences between treated and control samples, except for DHA content, which was significantly lower than control in treated strawberries, analysed soon after treatment (d0). As a consequence the redox status was higher in treated strawberries at d0, respect to control, indicating a less oxidized cellular redox state. DHA increased its content in treated sample at day d1 and d2, and the redox state of the two pools was then comparable. No significant differences were also noted between different storage times.

Table 5. Reduced ascorbic acid (AsA) and dehydroascorbic acid (DHA) content (mg/100g FW) determined in strawberry at harvest, immediately after AITC treatment (d0), and after one and two (d1 and d2) days of storage at 20°C. Redox status: AsA /(AsA+DHA) %. AITC treated sample means followed by an asterisk are significantly different from untreated control means (LSD test, P <0.05).

		Control	AITC
AsA	H	51.42 ± 4.62	
	d0	56.08 ± 5.13	55.38 ± 3.16
	d1	51.96 ± 3.94	53.69 ± 3.92
	d2	56.90 ± 3.19	52.64 ± 3.48
DHA	H	3.38 ± 1.04	
	d0	3.05 ± 1.58	1.55 ± 1.14*
	d1	3.21 ± 0.75	2.41 ± 1.05
	d2	3.71 ± 0.92	3.29 ± 0.71
AsA /(AsA+DHA) %	H	93.73	
	d0	94.82	97.37*
	d1	94.22	95.73
	d2	93.88	94.07

Glutathione content. Reduced and oxidised glutathione (GSH and GSSG) were determined at d0, d1 and d2, after storage at 20 °C of treated and untreated samples and results are shown in **Table 6**. The AITC determined a significant decrease of the reduced form of glutathione, GSH, soon after treatment (-89%). The GSSG content was also strongly reduced at d0, and the determined value for treated strawberry was very close to the assay limit of detection. Both parameters increased after one and two days of storage, but the GSH and GSSG content of treated samples were still more than 40% lower than control ones. The redox state seemed to be higher for treated samples respect to control at any sampling time, indicating a possible protective action of AITC against oxidative action, but the differences were not significant.

Table 6. Reduced glutathione (GSH) and oxidised glutathione (GSSG) content (nmol g⁻¹ FW) determined in strawberry at harvest, immediately after AITC treatment (d0), and one and two (d1) and d2) days of storage at 20°C. Redox status: AsA / (AsA+DHA) %. AITC treated sample means followed by an asterisk are significantly different from untreated control means (LSD test, P <0.05).

		Control	AITC
GSH	d0	54.7 ± 3.4	6.2 ± 5.0*
	d1	69.7 ± 10.1	42.1 ± 6.4*
	d2	67.2 ± 20.6	42.1 ± 6.8*
GSSG	d0	10.4 ± 1.6	0.3 ± 0.6*
	d1	7.8 ± 2.0	2.0 ± 1.0*
	d2	7.7 ± 3.4	3.5 ± 2.8
GSH/(GSH+GSSG)%	d0	84.00	95.82
	d1	89.8	95.5
	d2	90	93.1

3.3.1.4. Discussion and conclusions

The trial results showed the possibility of containing *B. cinerea* on strawberries by an alternative treatment to conventional fungicides, based on AITC, a natural compound released from *B. carinata* DSM. The *in vitro* efficacy of various ITCs, including AITC, against food-borne pathogens and spoilage bacteria was reported by many authors (Inatsu et al., 2005, Liu and Yang, 2010, Wilson et al., 2013), but there are fewer data on *in vitro* and *in vivo* activity of AITC and other ITCs against postharvest pathogens. In agreement with other authors that found mycelium more resistant than conidia towards natural fungicides (Fallik et al., 1998), the mycelium of *B. cinerea* was inhibited by AITC less than conidia (**Table 1**). In a previous work (Mari et al., 2008) the AITC EC₅₀ values were determined on *M. laxa* for conidia germination and mycelial growth, using the same methodology. Comparing these results, the EC₅₀ values for *M. laxa* conidia germination were lower than those found in this work, for *B. cinerea* (0.17 mg L⁻¹ against 0.62 mg L⁻¹), while the mycelial EC₅₀ resulted higher (1.90 mg L⁻¹ against 1.35 mg L⁻¹). Wu et al. (2011), instead, found lower AITC EC₅₀ values for conidia and mycelium of *B. cinerea* than those obtained in the present study (0.15 and 0.18 mg L⁻¹, respectively), but these differences could be explained by a different applied methodology, adopting 8000-mL desiccators instead of Petri dishes, in order to prevent AITC loss. AITC is a volatile compound and therefore different amounts of this substance could be lost during performance, despite the applied precautions.

Strawberries infected by *B. cinerea* in the field support latent infections that can spread during the postharvest phase, causing a high incidence of grey mould. In our experiments, in untreated ‘Tecla’ fruit, the incidence of *B. cinerea* was influenced by weather conditions with a percentage of

natural grey mould that varied between 44.7% (high disease level) and 13% (low disease level). Precipitations can play an important role in spore production and in inoculum dispersion. Frequent rains during the strawberry fruiting season can increase the splash dispersal of conidia, improving fruit infection (Maas, 1998). This could be a critical point for day-neutral strawberries, due to the longer production period and the later ripening period, and their disease management requires control approaches specific for each environmental condition not yet well investigated (Burlakoti et al., 2013). The efficacy of AITC against natural infections of *B. cinerea* on spring-bearing ('Tecla') and day-neutral ('Monterey') strawberries reported in our work, appeared promising, significantly reducing postharvest losses if compared to the control (**Table 2** and **3**). Few data have reported the activity of AITC on berries, while our results showed, for the first time, a significant control of a fungal pathogen in highly perishable fruit as strawberries. Wang et al. (2010) found a good reduction of decay development in blueberries treated with pure AITC ($5 \mu\text{L L}^{-1}$) and stored at 10°C for 21 days, even if the nature of decay was not specified. In addition, the AITC treatment dose, calculated from AITC added to the containers rather than the real AITC atmosphere concentration, appeared 50 times higher than the concentration applied in the present work.

AITC is generally classified as an antimicrobial substance active against a wide range of pathogens, including postharvest pathogens as *P. expansum* (Mari et al., 2002; Wu et al., 2011), and *M. laxa* (Mari et al., 2008). Other ITCs, assayed in similar trials, also showed an antifungal activity, such as benzyl-ITC vs *Alternaria alternata* on tomato (Troncoso-Rojas et al., 2005a), ethyl-ITC vs *P. expansum* on apple (Wu et al., 2011), butenyl-ITC vs *M. laxa* on peach (Mari et al., 2008) and a phenyl, benzyl, 2-phenylethyl and allyl-ITC mixture vs *Alternaria* rot on bell pepper (Troncoso-Rojas et al., 2005b).

In addition, previous results on the pathogen control obtained using AITC produced *in situ* from DSMs proved very scant. In the present work, synthetic and GL-derived AITC were compared and both controlled the disease with an efficacy index ranging from 47.4% to 91.5%. Previously, Troncoso-Rojas et al. (2005b) studied the potential of ITCs, including AITC, present in cabbage leaves to control *Alternaria* rot in bell pepper, but the treatments were based on a mixture of commercial reagent grade ITCs. In some cases, the ITCs were obtained from shredded, macerated or homogenized Brassica plant tissues (Mayton et al., 1996; Charron and Sams, 1999; Harvey et al., 2002), while in our experiments AITC was obtained from DSM, a co-product of *B. carinata* oil extraction developed with a biorefinery approach, as described in Lazzeri et al. (2013). The applied DSMs were formulated under patent (Lazzeri et al., 2010) to optimize the AITC releasing rate and consequently the biological potential activity on pathogens (section 3.2). This is a fundamental

aspect, showing that biofumigation could be optimized and used for industrial application. Moreover the use of bio-based chemicals obtained from renewable natural resources well fits with the European institutional goals, i.e. Regulation (EC) No. 1107/2009, Directive 2009/128/EC, COM (2012) 60 and the European Technology Platform 'Plant for the Future' (Mari, et al., 2011; De Nicola et al., 2013).

The influence of AITC on the level of antioxidant capacity and phytochemical content, was also investigated in 'Clery' strawberries, a variety very rustic and tolerant to diseases. Our results on antioxidant capacity, phenol and anthocyanin quantification showed no significant differences between treated and untreated fruit (**Table 4**), similar to those obtained on blueberries by Wang et al. (2010), thus suggesting that the AITC treatment had no harmful effect on fruit nutritional quality associated with this aspect. The treatment did not influenced also the final ascorbic acid content in the reduced, AsA, and oxidised, DHA, form, and the ascorbic acid redox state was unchanged between control and treated samples at the end of storage, d2 (**Table 5**). Anyway a lower level of DHA and a slight increase of the redox state in treated sample, soon after treatment, d0, seemed to indicate a protective action of AITC against oxidation, even if a physiological consequence of this transient effect, was not clearly evidenced on fruit. On the contrary a strong action of AITC on glutathione was observed in treated fruits, as it caused a significant depletion of GSH and GSSG, more evident at d0, but still significant at the end of storage. Nevertheless the redox status was not significantly affected by treatment (**Table 6**). The glutathione reversible depletion effect and consequent reactive oxygen species (ROS) accumulation has already been observed in AITC treated *Arabidopsis thaliana* seedlings, possibly indicating a role of the glutathione in the detoxification/control of ITCs in plant tissue in accordance with findings in mammalian cells (Øverby et al., 2015). Moreover, Wang et al. (2010) attributed the increased resistance of fruits to microbial infection after AITC treatment to a mechanism not related to antioxidant properties rather to the enhanced H₂O₂ production and hydroxyl radical formation in blueberries that resulted in an intolerable level of high oxidative stress on fungi cells. More investigations are needed to elucidate the AITC mechanism of action in fruit and its role on the generation of ROS.

Despite numerous data on ITCs antifungal, antibacterial, anti-nematode and anti-insect activities only a few studies have reported the analysis of residue content on treated fruits. AITC residues were found on fruit by the SPME technique (Arthur and Pawliszyn, 1990) coupled with GC-FID analysis, a solvent free, rapid and very sensitive technique that has become more and more popular in the extraction and analysis of organic and volatile compounds in food samples (Lambropoulou and Albanis, 2003; Vallverdù-Queralt et al., 2013). The concentration of GL-

derived AITC residue in fruit, treated 4 h at 0.1 mg L⁻¹, after 7 d from treatment, appeared lower than 1 mg kg⁻¹. Similarly, Mari et al. (2002) found less than 12 mg kg⁻¹ of AITC and no detectable residue in the fruit pulp, after 7 d at 20°C, in pear skin treated with 5 mg L⁻¹ (amount added to the treatment cabinet). AITC analysis of single fruit from different sampling points in the treatment cabinet, also showed a more homogeneous distribution of active molecule when GL-derived AITC was used than with the synthetic AITC (data not reported). This was probably due to the fact that, when DSM was used, AITC was produced in the reactor in vapour phase and then sent to the cabinet through a closed circle by a pump, with a consequently greater homogeneous active compound distribution. Synthetic AITC was instead injected as a liquid directly into the cabinet, thus creating concentration gradients in the cabinet. Regarding AITC safety, more detailed toxicological studies are needed. As a matter of fact, there is no AITC maximum residue limit (MRL) on strawberry. The MRL reported in the Regulation (EU) No 34/2013 for folpet, a fungicide applied against *B. cinerea*, is fixed at a limit of 3 mg kg⁻¹ of strawberry, a concentration higher than that observed after AITC treatment (**Table 3**). Overall, when assessing health risks, two key factors have to be considered: the levels where no health effects occur and the levels to which consumers may be exposed. Regarding the first key factor, AITC was defined as a flavouring substance which may be used as a foodstuff applying an acceptable daily intake (ADI) of 0.02 mg kg bw⁻¹ day⁻¹ (EFSA 2010). Taking into account the results reported in this paper, it can be calculated that to exceed the ADI a man of 60 kg should eat in a day more than 1.2 kg of strawberries treated as described. In addition, the strawberry taste should not be affected by AITC residues according to the results obtained by Ko et al. (2012) in assessing the sensory quality of Kimchi, a traditional Korean vegetable food, where an AITC concentration under 1.5 g kg⁻¹ did not affect the food taste.

In conclusion the reported results confirm even on strawberry the possibility of reducing the incidence of postharvest grey mould by over 45% with a biofumigation approach based on AITC, making this treatment promising for conventional and organic fruit production.

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3.3.2. Postharvest application of brassica meal-derived allyl-isothiocyanate to kiwifruit: effect on fruit quality, nutraceutical parameters and physiological response

3.3.2.1. Introduction

Kiwifruit is a fruit crop successfully adopted by international markets, with China, Italy and New Zealand being the top three producers worldwide (<http://faostat3.fao.org/browse/Q/QC/E>). Despite the spread of new varieties developed from different species, the most popular cultivar is the green-fleshed ‘Hayward’ (*Actinidia deliciosa*), whose distinctive appearance and flavour are highly appreciated by consumers. The great economic interest in kiwifruit is linked to its high nutraceutical values, together with the long-term storage capability and consequent exportability. After harvest, fruits are usually stored at 0°C under controlled atmosphere (CA), where the removal of ethylene increases the storage time up to 4 months, preserving both fruit quality and flavour (Latocha et al., 2014). Kiwifruits are an excellent source of minerals (e.g. potassium), dietary fibres, and bioactive compounds, such as vitamins E and C (L-ascorbic acid, AsA), flavonoids, anthocyanins, and carotenoids (Drummond, 2013). *A. deliciosa*, in particular, is rich in ascorbic acid and polyphenols that contribute to the total antioxidant capacity and to the capability of limiting oxidative stress due to reactive oxygen species (ROS). These properties make kiwifruit a ‘functional food’ with preventive effects against certain types of cancers, vascular diseases and gastro-intestinal disorders (Padmanabhan and Paliyath, 2016). In plants, ROS are involved in fruit senescence, as excessive production causes lipid peroxidation and membrane damage, but they are also response factors for the activation of defence mechanisms after abiotic and biotic stress. Whether they act as toxic or signalling factors in response to environmental changes depends on the balance between their production and scavenging, and a complex network of mechanisms is involved in ROS homeostasis. L-ascorbic acid and glutathione are the main key players involved in this process, the first as direct antioxidant or as cofactor of enzymes participating in the biosynthesis of secondary metabolites and plant hormones, the second with a primary role in xenobiotic detoxification, redox balance, plant development and defence (Foyer and Noctor, 2011). Together with those non-enzymatic antioxidant compounds, enzymes as catalases (CAT), superoxide dismutases (SOD) and peroxidases (POD) are also involved in ROS scavenging and play an important role in fruit physiological changes during ripening and stress response (Xia et al., 2016). Besides, even though kiwifruits contain low levels of proteins, two of the most abundant soluble proteins (actinidin, a cysteine proteinase and a thaumatin-like protein, PR5) can affect fruit quality and have beneficial effects on humans. In particular, actinidin contributes to the digestive process, while PR5 is known as a sweetener and flavour modifier, inducible by various agents from ethylene

to pathogens. Nonetheless, several pre and postharvest factors, such as the physiological state of the fruit at harvest time, genotype, cultivation techniques, storage period and temperature management, can greatly affect both qualitative and nutritional aspects of kiwifruits (Tavarini, et al. 2008, Oz, 2010). Among postharvest techniques, the use of natural preservatives, as plant secondary metabolites, could represent a valid option, within an integrated management, for the shelf-life extension of goods. Allyl-isothiocyanate (AITC) demonstrated a strong antimicrobial activity on fruit as strawberries (section 3.3.1), while the effect on plant and the mechanism of action at cellular and molecular level is still poorly studied. At the best of our knowledge, there are no literature data on the AITC effect on the nutraceutical characteristics of kiwifruit. This work was therefore focused on the evaluation of the effects of an AITC treatment on kiwifruit physico-chemical parameters, antioxidant enzymes, glutathione content, expression of genes involved in ripening, stress response and secondary metabolism, in order to assess the potential application of the treatment in improving fruit quality and nutraceutical properties.

3.3.2.2. Materials and methods

Plant material

Kiwifruits (*Actinidia deliciosa*, cv. 'Hayward') were harvested at commercial maturity stage from an orchard located in the production district of Emilia Romagna Region (Italy) after a management by the Italian integrated production guidelines. Fruits homogenous in maturity stage and size were carefully selected, with the aim of excluding those fruit with physical injury, diseases, or incidences of pests.

Isothiocyanate

Allyl-isothiocyanate was produced *in situ* from the endogenous GL (sinigrin) - MYR system of *Brassica nigra* defatted seed meal (DSM), provided by Agrium Italia SpA (Livorno, Italy) and opportunely formulated (Lazzeri et al., 2010). DSM used for the trials had defined characteristics as 5.1 % moisture content, 7.9 % residual oil and sinigrin content of 153 $\mu\text{mol g}^{-1}$ (on dry matter, DM) determined with standard procedures (Franco et al., 2016).

AITC treatment

Selected kiwifruits were distributed in 3 trays (26 fruits each), placed in a 0.1 m³ airtight cabinet and treated by AITC vapours produced by the DSM plus water added in an external reactor as described in Mari et al., (2008) (section 3.1). Allyl-isothiocyanate concentration was maintained at 0.15 mg L⁻¹ for 5 hours. The atmosphere in the cabinet was monitored by an Agilent 7820AC GC system equipped with an Agilent HP-5 column (30 m length, 0.32 mm inner diameter, 0.25 μm film

thickness) and FID detector (Agilent Technologies, Santa Clara, USA). Injector and detector temperatures were set at 200°C and 300°C respectively; the oven program started at 60°C and raised to 120°C at a rate of 10°C min⁻¹; the carrier gas (He) flow rate was 1 mL min⁻¹ and the splitless injection mode was selected. The AITC amount was determined on the basis of a previously defined calibration curve using a pure AITC standard (Sigma-Aldrich, Saint Louis, USA). Treated fruits and non-treated control fruits were stored at 12-15 °C overnight and then for 120 days in CA (2% O₂, 4.5% CO₂) at 0°C and 95% relative humidity, maintaining ethylene concentration <0.02 µL L⁻¹ by scrubbing the gas with KMnO₄. Six to ten fruits were randomly sampled per each time point: i) at harvest, ii) at the end of the treatment (d0), iii) one day after the treatment (d1) and iv) at the end of storage (d120). All analyses were performed on three pools of fruits.

Kiwifruit quality traits

Quality trait evaluation was performed on fruits at harvest at d120. Kiwifruits were weighed and peeled, and the homogenised samples were analysed for:

Soluble solid content (SSC), determined by a digital refractometer (Refracto 30 PX, Mettler Toledo, Milan, Italy), (expressed as °Brix);

Total titratable acidity (TA), measured by titration of kiwi juice acids with 0.1 N sodium hydroxide (expressed as meq L⁻¹);

pH value, measured using a digital pH-meter (785 DMP, Methrom, Milan, Italy);

Firmness, measured on fresh fruit by a penetrometer (Fruit Pressure Tester FT011, TR snc, Forlì, Italy), using a 10 mm tip (expressed as Newton, 1 N = 9.8 kg);

Moisture content (unbound water) measured by weight loss after drying fresh samples at 105° ± 1 °C, until a constant weight was reached (expressed as % dry matter, DM).

Extraction and analysis of phytochemical content and antiradical capacity of kiwifruits

A mixed sample of fresh fruits at harvest and at d120 were peeled, cut into small pieces, freeze-dried and stored at -80°C until analysis. Freeze-dried samples (0.5 g) were extracted with 10 mL of a solution of methanol: water (80:20 v/v) acidified with 0.1% HCl (v/v) to determine fruit phytochemical content and antiradical capacity (AC). The extraction was carried under magnetic stirring (300 rpm), for 30 min, at room temperature in the dark. The mixture was then submitted to an ultrasound-assisted extraction for another 30 min (40 kHz, 10°C) and centrifuged at 6792 g, for

15 min at 4 °C. After a second extraction, supernatants were collected together and immediately analysed.

Total polyphenol content (TPC) and *flavan-3-ol content (FLC)* of extracts were determined according to Carbone and Mencarelli (2015). Data were expressed as mg of gallic acid equivalents (GAE) and mg catechin equivalents (CAE) per g on DM for TPC and FLC, respectively.

L-ascorbic acid content (AsAC) of kiwifruits was determined by Differential Pulse Voltammetry (DPV) and Cyclic Voltammetry (CV). Briefly, the DPV measurements were performed using a computer-controlled system, AUTO-LAB model PGSTAT 12 with GPES software (ECONCHEMIE, the Netherlands). Voltammetric measurements employed a screen-printed electrode produced in house, which consisted of a three electrode configuration with graphite working and counter electrodes and a silver pseudo-reference electrode printed onto a plastic support film. All measurements were carried out in 0.1 M KCl aqueous solutions at room temperature. The DPV and CV were performed according to Cavalieri et al. (2013) using 60 µL of AsA standard solutions and extracted fruit samples in order to observe the electrochemical behaviour of each analyte and the matrix effect. The quantitative determination of AsA in kiwifruits was extrapolated using the calibration curves obtained with increasing amounts (0 – 125 x 10⁻⁶ M) of AsA (standard solutions) in 0.1 M KCl using DPV.

Antiradical capacity (AC) was assessed measuring the ability of the extracts to scavenge synthetic radicals [i.e. 2,2-diphenyl-1-picryl-hydrazyl-hydrate, DPPH• and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), ABTS•⁺]. Both DPPH• and ABTS•⁺ quenching capacities were estimated spectrophotometrically according to Carbone and Mencarelli (2015), and expressed in terms of EC₅₀.

Antioxidant enzyme activity assays

Fruit pericarps, collected at d0 and d1, were cut, frozen, grinded in liquid nitrogen by a Waring Commercial laboratory blender (Waring products division Torrington, CT, USA) and stored at - 80 °C. Enzyme extraction and analysis were performed according to Yang et al. (2013), unless otherwise stated. Samples were homogenised with 0.5 M potassium phosphate (pH 7.0), 1 mM ethylene diamine tetraacetic acid (EDTA), 50 g L⁻¹ polyvinylpyrrolidone and 10 mL L⁻¹ Triton X-100. After centrifugation (31.500 g, 20 min, 4°C), the supernatant was used for enzyme spectrophotometric assays carried out in a Varian Cary 300 Bio Uv/Vis spectrophotometer (Agilent Technologies).

Catalase (CAT) activity was determined by monitoring the decrease of absorbance at 240 nm of the reaction mixture containing 50 mM phosphate buffer (pH 7), 10 mM of H₂O₂ and the kiwifruit extract.

Guaiacol peroxidase (POD) activity was determined by monitoring the increase in absorbance at 470 nm of the mixture composed by 50 mM potassium phosphate (pH 7), 20 mM guaiacol (Sigma-Aldrich), 10 mM H₂O₂, and kiwifruit extract.

One unit (U) of CAT or POD activity was defined as the amount of enzyme causing a change in absorbance of 0.01 min⁻¹.

Superoxide dismutase (SOD) activity was determined by using an Infinite M200 PRO microplate reader (Tecan Austria GmbH). The reaction mixture containing 50 mM potassium phosphate (pH 7.8), 13 mM methionine, 75 µM nitrobluetetrazolium (NBT), 2 µM riboflavin (Sigma-Aldrich), 0.1 mM EDTA and enzyme extract was exposed 10 min to light from a fluorescent lamp (Sylvania F15T8-CW Cool White fluorescent lamp). The absorbance was read at 560 nm and subtracted from blank samples with and without kiwifruit extracts, illuminated or not. One U of SOD was defined as the amount of enzyme that inhibits the photoreduction of NBT by 50% under the assay conditions. Specific enzyme activities were expressed as U g⁻¹ protein. Extract protein concentration was determined using the Bio-Rad dye reagent (Bio-Rad, Munchen, Germany).

Glutathione content determination of kiwifruit

Frozen ground pericarp samples collected from fruit at d0 and d1 were extracted with cold 5% 5-sulfosalicylic acid (SSA), 1:10 w/v, at 4°C by using a TissueLyser (Retsch GmbH, Haan, Germany), and subsequently centrifuged at 31,500 g for 15 min at 4°C. Total glutathione (reduced + oxidized glutathione, GSH + GSSG) spectrophotometric analysis was performed by using the GSH assay kit (Sigma-Aldrich), with GSH reductase, 5,5'-dithiobis(2-nitrobenzoic acid (DTNB) and NADPH, according to the provided protocol on a Infinite M200 PRO microplate reader. GSSG was determined by the same method in presence of GSH masking agent 1-methyl-2-vinylpyridinium triflate (M2PV) (Shaik and Mehvar, 2006). Results are expressed as nmol g⁻¹ FW. GSH was estimated as the difference between total and oxidized form and GSH redox state calculated as the percentage of GSH over total: $GSH/(GSH+GSSG) \times 100$.

Gene expression analysis: RT-qPCR

Total RNA was isolated from 200-300 mg of frozen kiwifruit outer pericarp collected at d0 and d1, following the protocol of Chang et al. (1993), scaled down in order to work in 2 mL tubes. RNA was then cleaned up using MinElute column and on column DNase I treatment (Qiagen),

according to manufacturer's instructions. RNA quantity and quality were evaluated with the Infinite 200-PRO NanoQuant system (Tecan, Männedorf, Switzerland) and on 1% agarose gel, respectively. cDNA synthesis was performed starting from 500 ng of RNA in 10 µL reactions, using High-Capacity RNA-to-cDNA Kit (Life Technologies). Gene-specific primers (**Table 1**) were chosen from literature or designed using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi, Huang et al. 2013) from EST sequences available at NCBI or gene predictions available at the Kiwifruit Genome database (<http://bioinfo.bti.cornell.edu/cgi-bin/kiwi/home.cgi>).

Table 1 List of the primers used for transcript abundance analysis by RT-qPCR, with respective gene identification code (ID) from the Kiwifruit Genome Database (<http://bioinfo.bti.cornell.edu/cgi-bin/kiwi/search.cgi>, or GeneBank database (<https://www.ncbi.nlm.nih.gov/genbank>). The gene type (reference or gene of interest, GOI) and the metabolism in which GOIs are involved are specified. Superscript letters indicate the reference of the primers' sequences: a: Günther et al., 2011, b: Richardson et al., 2011, c: Li et al., 2010.

Short name	Gene description	Type / metabolism	Primer forward 5'->3'	Primer reverse 5'->3'	Gene ID
<i>TUA</i>	alpha-tubulin	reference	ACAACACAGCAGTGGCAGAG	AGAGAACTCCCCTTCCTCCA	FG466654
<i>UBC9</i> ^a	ubiquitin conjugating enzyme	reference	CCATTTCCAAGGTGTTGCTT	TACTTGTTCCGGTCCGTCTT	FG524028
<i>UBQ</i>	ubiquitin	reference	CCAAGATCCAGGACAAGGAG	TGGAGGGTCGATTCTTTCTG	FG409945
<i>SAM</i>	S-adenosylmethionine synthase	GOI/Ethylene synthesis	CCCAGATTTCACTTGGGAGA	TCTTCCTCAGCCAGAATGGT	ACU17240
<i>ACO1</i>	ACC oxidase 1	GOI/Ethylene synthesis	CCGCTTGTGTTTGTGTGAAC	TTCTTTCTGGGGAATGCAAC	HQ293204
<i>ETR2</i>	ethylene receptor	GOI/Ethylene Perception	AAAACGTGTGGGAAAGATGC	CAATCCCGTCATCACACAAC	EU170629
<i>PG</i>	polygalacturonase	softening/Cell wall degradation	GTTAGGGGTGTGCTCTTCCA	GATTGGGGCAATTTTTGTTG	AF152756
<i>BAM</i> ^b	beta-amylase	GOI/Fruit ripening/starch degradation	ATCCTGACCTTGTCTACACA	ATGGGTGTTCTTCTCTAAG	FG525163
<i>PAL</i>	phenylalanine ammonia-lyase	GOI/Phenyl propanoid pathway	GCTTGGATTACGGGTTCAAA	GAGTTCACATCTGGTTGTGTTG	Achn153791; Achn066341
<i>CHS3</i>	chalcone synthase	GOI/Phenyl propanoid pathway	AGAGAAGTTCAGCGCATGT	ATTTGGGAATTTCCACCACA	Achn168721
<i>PPO</i>	polyphenol oxidase	GOI/Stress response	GGTGATCCGACCTGACAAC	ACAGCTCCGTGATACCCAAC	Achn226891
<i>GPP</i>	L-galactose-1-phosphate phosphatase	GOI/Ascorbic acid synthesis	TACCAAACCAAGCATGTGGA	ACTTGTGGTCAGGAAATGC	AY87585
<i>GalDH</i> ^c	L-galactose dehydrogenase	GOI/Ascorbic acid synthesis	TTGGGAAGGATGCGAAAAC	GCAGCCACTTCACAAAGCA	EU525846
<i>MDHAR</i> ^c	monodehydroascorbate reductase	GOI/Ascorbic acid recycling	TCAAATTATCAAGGAACTGTGGC	ACCAACAACAACGATGTCCGCT	Achn091771; Achn075231; GU339041
<i>GST</i>	tau class glutathione transferase 5	GOI/Glutathione metabolism	TTGCTGGAGCAAAGACAATG	GGCTTCTGAATGATTCCAA	Achn223211
<i>Act d1</i>	actinidin	GOI/Abundant protein in kiwifruit	AGGCTACATGAGGATCCTGC	GGCCATCCTTGCTCATTGAG	M38422
<i>PR5</i>	thaumatin-like protein	GOI/Abundant protein in kiwifruit	TGATGGGTTCATGTGGCGA	CCAGGGGCACGTAACCTCATT	AJ871175

qPCR was performed using the Rotor-Gene 6000 Instrument (Corbett Life Science) and Sybr Select Master Mix (Life Technologies) with 100 nM specific primer pairs and 2 μ L of 1:10 diluted cDNA, in 15 μ L total volume. After the initial denaturation at 95°C for 2 min, 45 amplification cycles were performed (95°C for 15 sec, 60°C for 60 seconds), followed by melting curve analysis. Each analysis was repeated twice. *UBQ*, *UBC9*, and *TubA* were used as reference genes. Calculations were made according to the $2^{-\Delta\Delta C_t}$ method using REST software (Pfaffl et al., 2002).

AITC residue analysis in kiwifruits

Residual AITC quantification was performed on kiwifruits at d120. Fruits were peeled and 0.5-1 g of the epicarp (2-3 mm thick) or 5 g of the outer pericarp were separately cut into small pieces and analysed by solid phase microextraction (SPME) coupled with GC-FID technique as described in section 3.3.1. GC analysis was carried out on a Varian GL-3800 equipped with an Agilent HP-5 column (30 m, 0.25 mm, 0.25 μ m), an automatic auto-sampler CombiPAL (CTC Analytics) and FID detector. The analytical conditions were: injector and detector temperature maintained at 260 °C and 280 °C respectively; flow of the carrier gas (nitrogen) set at 1 mL min⁻¹; column oven temperature followed a gradient from 60 °C to 90 °C, with a rate of 5 °C min⁻¹ and from 90 to 200 °C with a rate of 40 °C min⁻¹. The splitless mode was used for injection. Blank samples of untreated fruit were analysed following the same protocol. A calibration curve with blank sample of the non-treated epicarp and the outer pericarp spiked with AITC hexane dilutions was constructed and the limit of quantification and detection (LOQ and LOD) were determined. Measurements were replicated 5 times for treated fruits and calibration samples.

Statistical analysis

Described analysis were carried out in triplicate and statistical analysis of data expressed as mean \pm standard error was performed with the SPSS 17.0 software (SPSS, Inc., Chicago, Illinois), unless differently specified. Data were then subjected to a one-way analysis of variance (ANOVA) employing Tukey's HSD test to assess significant differences between samples analysed ($P < 0.05$). Finally, Pearson's correlation coefficient (r) was used to determine variable correlations ($P < 0.01$).

3.3.2.3. Results and discussion

Effect of AITC on kiwifruit quality traits, phytochemical content and antiradical capacity

The mean values of the physicochemical attributes of kiwifruit at the end of storage are shown in **Table 2**.

Table 2 Pomological and nutraceutical parameters (mean \pm SE) of kiwifruits treated or not with AITC and determined at the end of storage (d120). Results are expressed on a dry matter (DM) basis. DM (%), soluble solid content (SSC, °Brix); titratable acidity (TA, meq L⁻¹); firmness (N); ascorbic acid content (AsAC, $\mu\text{g g}^{-1}$); total phenolic content (TPC, mg gallic acid equivalents GAE g⁻¹); total flavanol content (FLC, mg catechin equivalents CAE g⁻¹); EC₅₀_DPPH• (mg)^a; EC₅₀_ABTS•⁺ (mg)^a. AITC treated (AITC) sample means followed by an asterisk are significantly different from untreated control means ($P < 0.05$, Tukey's HSD test).

	Control	AITC
DM	14.9 \pm 0.4	14.1 \pm 0.2
SSC	13.3 \pm 0.1	12.93 \pm 0.03*
TA	258 \pm 1	261 \pm 2
pH	3.43 \pm 0.02	3.38 \pm 0.02
Firmness	31.5 \pm 0.3	33.8 \pm 0.2
AsAC	69 \pm 2	105 \pm 4*
TPC	3.078 \pm 0.009	3.48 \pm 0.03*
FLC	1.398 \pm 0.007	1.505 \pm 0.002*
EC₅₀_DPPH•	12.56 \pm 0.01	10.0 \pm 0.1*
EC₅₀_ABTS•⁺	1.029 \pm 0.004	0.86 \pm 0.01*

^aEC₅₀ = mg of kiwifruits required to obtain 50 % DPPH• or ABTS•⁺ scavenging

All fruits were at an adequately ripe stage for consumption (SSC \geq 12.5 °Brix) (Beirão-da-Costa et al., 2008), with no decay. Postharvest AITC treatment did not significantly influence the overall quality of kiwifruits except SSC, which decreased in the treated samples as compared to the control after cold storage ($P < 0.05$). These findings agree with those reported in the literature on the ability of AITC in maintaining the postharvest fruit quality (Wang, 2003).

Kiwifruits are generally recognised as a valuable source of vitamin C. However, this is a readily degradable compound, so it is fundamental to know how postharvest technologies could affect its content to identify the treatments that reduce AsA losses in ripe fruit. In this study, kiwifruit AsAC was markedly affected by AITC treatment (**Table 2**). In fact, AsAC strongly decreased with storage (- 55% in control fruits) as already observed by Oz (2010), while AITC application significantly reduced its losses, keeping the level similar to those of samples at harvest time (152 \pm 6 $\mu\text{g g}^{-1}$ DM). Kiwifruits are also known for the presence of other valuable compounds as polyphenols (Du et al., 2009). In this study, storage markedly affected even the total polyphenol content (TPC) of analysed samples. The highest TPC was found in AITC treated kiwifruits, which was 24% higher compared to that at harvest time (2.810 \pm 0.007 mg GAE g⁻¹ DM; $P < 0.05$) and also significantly higher than control samples (+ 13%). FLC was also increased by AITC treatment, which produced a significant accumulation of flavans compared to the control samples (~ + 8%). In

this work the antioxidant potential of kiwifruits was also evaluated by means of two different *in vitro* assays: the DPPH• and ABTS•⁺ scavenging methods. The results are expressed as the amount of antioxidant able to reduce the initial radical concentration by 50%, a value generally defined as EC₅₀ (**Table 2**): the higher the antiradical capacity, the lower this value is. In both assays, AITC treated samples showed the highest AC, which was 1.6-fold higher than that at harvest (EC₅₀ = 15.75 ± 0.08 mg on DM; EC₅₀ = 1.39 ± 0.03 mg on DM, for DPPH• and ABTS•⁺ scavenging, respectively). Parametric correlation analysis pointed out a very good negative correlation between TPC and AC (r = -0.96 and -0.89, for DPPH• and ABTS•⁺, respectively) significant at 0.01 level (2-tailed). AC also showed a good negative correlation with AsAC, which was higher in the case of ABTS•⁺ radical scavenging (r = -0.59 and -0.75, for DPPH• and ABTS•⁺, respectively; p < 0.01, 2-tailed). These findings are in agreement with those reported in the literature (Du et al., 2009), highlighting the contribution of vitamin C and polyphenols to the antioxidant potential of kiwifruits. The results show for the first time the AITC effect of prevention of AsAC and TPC loss on kiwifruits during postharvest storage in AC. The same effect was not observed in strawberries (Section 3.3.1), also probably because of the different storage conditions of the fruit (subjected to short storage) compared to the AC long storage of kiwifruit, besides the overall different fruit characteristics. The exact mechanism of action of the AITC is not known and investigations by biochemical and gene expression analysis were performed in kiwifruits as described below in order to have some indication on the possible metabolic pathways and enzymes to be involved.

Effect of AITC on antioxidant enzyme activity and glutathione kiwifruit content

The short- and long-term effect of AITC on the activity of antioxidant enzymes in kiwifruits was investigated. The results (**Table 3**) highlighted a significant increase of SOD activity in treated kiwifruits compared to untreated ones, determined immediately after the treatment (d0). The basal level of activity was quickly restored at 24 h (d1) and no differences were detected at the end of storage (d120). Regarding CAT and POD activities, these were similar to those found by other authors for kiwifruits stored 120 days in cold storage (Yang et al., 2013), but no significant differences were evidenced between AITC-treated and control kiwifruits as between fruits before and after storage. SOD is the first line of defence against ROS toxicity and is a strong free radical scavenging enzyme that neutralizes the superoxide radical into the less toxic hydrogen peroxide which can be further converted into water by CAT and POD enzymes (Xia et al., 2016). It can be speculated that SOD increased activity might play a protective role against an initial burst-like effect of AITC treatment. This hypothesis was further validated by reduced and oxidized glutathione quantitative determination. Soon after AITC application (d0) reduced glutathione

(GSH) content was significantly lower (-16%) in treated compared to control samples, while oxidized glutathione (GSSG) content was higher in AITC treated samples, even if not significantly. After 24 hours (d1), GSH and GSSG levels increased and decreased respectively in both treated and control, and the redox state of the two pools was comparable. This effect was also observed for AITC treated strawberries (0.1 mg L⁻¹, 4 h treatment) as described in section 3.3.1. where the GSH depletion was even higher (-89%). Nevertheless a strong decrease of GSSG was also noted in strawberry. Furthermore in strawberries the GSH and GSSG AITC depletion effect was not recovered at 100% after only two days of storage.

Table 3 Antioxidant enzyme activities (Unit mg⁻¹ of protein) and glutathione content (nmol g⁻¹ FW) determined in kiwifruit immediately after AITC treatment (d0), one day later (d1) and after 120 days of storage under controlled atmosphere (d120): superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), reduced glutathione (GSH), oxidized glutathione (GSSG), redox state [GSH/(GSH+GSSG)%]. Untreated control and AITC treated (AITC) sample means followed by the same letters are not statistically different (P <0.05, Tukey's HSD test).

		Control	AITC
SOD	d0	30.8 ± 2.4 b	40.2 ± 4.5 a
	d1	29.6 ± 3.4 b	31.2 ± 1.7 b
	d120	34.6 ± 5.5 ab	32.4 ± 5.7 ab
POD	d0	12.9 ± 1.7 a	17.7 ± 4.5 a
	d1	17.9 ± 3.5 a	17.3 ± 5.7 a
	d120	22.0 ± 2.6 a	15.8 ± 10.4 a
CAT	d0	1.4 ± 0.6 a	1.7 ± 1.2 a
	d1	1.5 ± 0.7 a	1.4 ± 0.5 a
	d120	1.3 ± 0.3 a	1.3 ± 0.5 a
GSH	d0	158.2 ± 10.5 a	133.3 ± 11.9 b
	d1	171.3 ± 25.7 a	153.1 ± 8.7 ab
GSSG	d0	6.4 ± 1.0 a	8.1 ± 1.0 a
	d1	2.9 ± 0.4 b	2.7 ± 0.1 b
GSH/(GSH+GSSG)%	d0	96.1 b	94.3 c
	d1	98.3 0.2 a	98.3 0.2 a

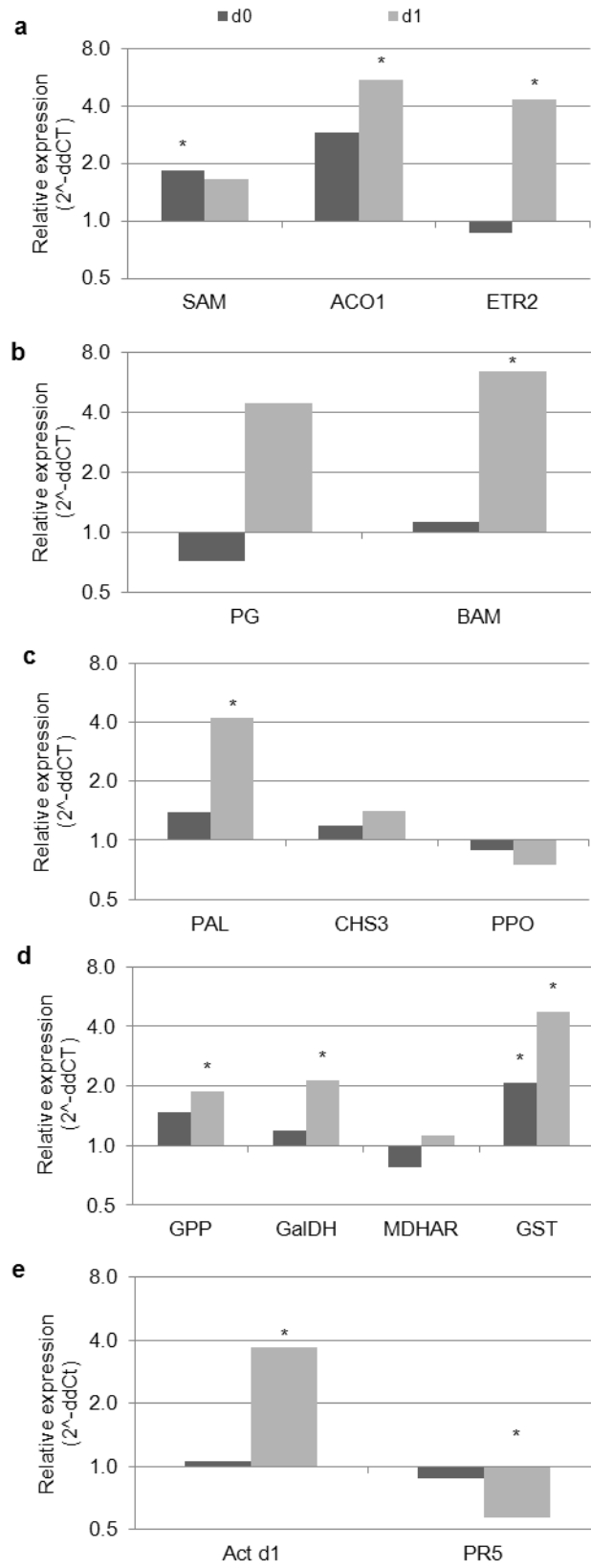
AITC treated kiwifruit samples showed a more oxidized cellular redox state than control immediately after treatment, suggesting an initial but reversible oxidative stress caused by AITC treatment. On the contrary, in strawberries, the redox state was not significantly affected by treatment at any sampling time. As already mentioned the glutathione reversible depletion effect and consequent ROS accumulation has already been observed in AITC treated *Arabidopsis thaliana* seedlings, possibly indicating a role of the glutathione in the detoxification/control of ITCs in plant

tissue in accordance with findings in mammalian cells (Øverby et al., 2015). Total glutathione level was also rapidly recovered after a few hours in *Arabidopsis*. Lower GSH content after AITC postharvest treatment has also previously been observed in blueberries and raspberries (Chanjirakul et al., 2006; Chen et al., 2015). The GSH depletion effect seems to be dose and time dependent and strawberries are probably more susceptible than kiwifruit due to their very different physiological and morphological characteristics. Anyway although it is established that AITC conjugates with GSH in causing oxidative stress, it remains unclear whether the consequent GSH depletion is linked to a physiological response in plant. GSH/GSSG is the major cellular redox buffer along with ascorbate/dehydroascorbate and changes in these ratios can reflect cellular toxicity but have also been associated with important redox signalling and regulation of gene expression related to stress defence. In kiwifruit the AITC effect on the redox state could be correlated with the induction of protective and antioxidant non-enzymatic systems and could be responsible for the increased AsA, polyphenol and flavan content in AITC treated fruit (**Table 2**), even though this hypothesis needs further investigation.

Gene expression analyses

The expression level of 14 genes involved in different metabolisms related to fruit ripening and stress responses (as detailed in **Table 1**) was analysed in response to AITC application, immediately after treatment (d0) and one day later (d1). Results are shown in **Figure 1**.

Figure 1. Relative transcript abundance by RT-qPCR of genes belonging to different metabolisms in kiwifruits treated with AITC at the end of the treatment (d0) and 24 hours afterwards (d1). a) ethylene synthesis and perception. SAM: S-adenosylmethionine synthase, ACO1: ACC oxidase 1, ETR2: ethylene receptor; b) Cell wall and starch degradation. PG: polygalacturonase, BAM: beta-amylase; c) Phenyl propanoid pathway and phenol oxidation. PAL: phenylalanine ammonia-lyase, CHS3: chalcone synthase, PPO: polyphenol oxidase; d) Ascorbate/glutathione biosynthesis and metabolism. GPP: L-galactose-1-phosphate phosphatase, GalDH: L-galactose dehydrogenase, MDHAR: monodehydroascorbate reductase, GST: tau class glutathione transferase 5; e) others. Act d1: actinidin, PR5: thaumatin-like protein. Each bar represents the ratio between expression in AITC treated fruits and their untreated control at the specified time point. The results were normalized to multiple reference genes and expressed as $2^{-\Delta\Delta C_t}$. Stars indicate significant difference according to REST software analysis



Since the reported qualitative results on kiwifruits indicated a reduced loss of AsA after AITC treatment during postharvest storage (**Table 2**), it was decided to investigate two genes involved in AsA biosynthesis through the main L-galactose pathway (L-galactose-1-phosphate phosphatase *GPP* and L-galactose dehydrogenase *GaldH*) and one (monodehydroascorbate reductase *MDHAR*) involved in AsA recycling. The increased expression levels of *GPP* and *GaldH* suggest that AITC might compensate for AsA degradation through *de novo* biosynthesis. *MDHAR*, instead, was not affected at all by AITC. The transcriptional behaviour of a tau class glutathione transferase-encoding gene (*GST*) was also assessed in this work as linked to AsA through the glutathione-ascorbate cycle. *GST* enzymes have functions in detoxification as they catalyse the conjugation between GSH and electrophilic xenobiotics and their activity is often up-regulated in response to various types of stress. Øverby et al. (2015) in *A. thaliana* demonstrated that AITC is not only involved in a reversible depletion in GSH-pool, as mentioned above, but also in the elevated expression of *GST*-encoding genes. In agreement with this report, *GST* gene was up-regulated upon AITC treatment. The different GSH redox state observed early in treated fruit (**Table 3**) could be part of the signalling transduction induced by AITC that influenced the expression levels of these genes.

In kiwifruits, contrary to tomato and many other climacteric fruits, most physiological changes associated to ripening occur before autocatalytic ethylene production is detectable and ethylene production is associated more to fruit softening and senescence than ripening. In this work, three genes potentially involved in ethylene production and perception (S-adenosylmethionine synthase *SAM*, 1-aminocyclopropane-1-carboxylate oxidase 1 *ACO1* and ethylene receptor 2 *ETR2*) were found either slightly increased or significantly up-regulated in response to AITC, suggesting the activation of ethylene synthesis and perception. *SAM* synthase genes and enzymes and ethylene receptors have previously been shown to be ethylene-responsive in kiwifruits (Atkinson et al., 2011), therefore AITC could initiate an autocatalytic process of ethylene biosynthesis and response that would normally occur later. It is known that the expression of beta-amylase (*BAM*) is positively linked to this process and associated to starch degradation and soluble sugar accumulation. Consistently, in our system *BAM* and polygalacturonase *PG*, which is involved in cell wall degradation, were induced one day after treatment, suggesting that cell wall remodelling and soluble sugar accumulation, naturally occurring in kiwifruit during late stages of fruit ripening, would be anticipated by AITC treatment. However, this did not affect final fruit firmness (**Table 2**), probably because this process was stopped by subtracting ethylene during CA storage.

Phenolic secondary metabolites affect plant-derived food quality characteristics such as appearance, flavour and other health-promoting properties. Their content in foods is affected by many factors that influence phenolic stability, biosynthesis and degradation. In terms of biosynthesis, the key enzyme phenylalanine ammonia-lyase (PAL) is especially relevant, as it can be induced by different stress conditions and ethylene itself can induce both the gene expression and the enzyme activity of *PAL* (Singh et al., 2010). In this work, a 4x induction of *PAL* expression level was observed, probably responsible for the significantly higher TPC value in kiwifruit at the end of storage (**Table 2**), even though the tested chalcone synthase gene (*CHS3*), a downstream gene in the flavonoid biosynthetic pathway, was not affected by the treatment. Moreover, a polyphenol oxidase (*PPO*), encoding one of the main enzymes responsible for fruit quality loss due to phenolic degradation, was down-regulated.

Finally, the expression level of two-gene coding for the most abundant proteins in kiwifruit was also investigated, actinidin (*Act d1*) and a thaumatin-like protein (*PR5*). Both genes were sensitive to AITC, though with divergent trends of modulation. Interestingly, *Act d1* was three times more expressed in AITC treated samples than in control ones. This could lead to an increased concentration in actinidin, which generally decreases during fruit ripening, contributing to further enhancement of nutritional values and shelf-life of the fruit. The opposite was found for *PR5*, although further studies are required to understand if this could impact on fruit quality.

AITC residues in treated kiwifruits

After a 4-month storage period of kiwifruits, the concentration of AITC residues in the epicarp was below the LOQ of 0.26 mg Kg⁻¹, corresponding to about 0.075 mg Kg⁻¹ of the whole fruit, and no detectable residues were found in the outer pericarp (LOD = 0.0096 mg Kg⁻¹, corresponding to about 0.0024 mg Kg⁻¹ of the whole fruit). According to EFSA, the acceptable daily intake (ADI) for AITC used as food additive is 0.02 mg/Kg bw/day (EFSA, 2010), so in our case the residues are largely below this limit, even in the case of presumable use of freeze-dried kiwifruit pulp extracts.

3.3.2.4. Conclusions

A 5-hour treatment with 0.15 mg L⁻¹ AITC in combination with CA can preserve 'Hayward' kiwifruit quality and enhance the nutraceutical properties in terms of increased antiradical capacity and phytochemical content, without leaving any detectable AITC residues on fruit. GSH cycle involvement in AITC mechanism of action/detoxification was confirmed in kiwifruits and AITC influence on AsAC and ethylene synthesis and PAL was depicted at gene expression level. These

findings open new perspectives in the use of this natural compound to enhance phytochemical content and antioxidant properties of fresh and, potentially, minimally processed fruits.

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CHAPTER 4.

OTHER DEFATTED SEED MEAL APPLICATIONS

4.1. *Eruca sativa* and industrial crops: a wealth of active ingredients for the health-food industry

Growing evidences demonstrate that a correct Mediterranean Diet is associated with a significant reduction in total mortality (Estruch et al., 2013). The dominant component of this diet as predictor of lower mortality are moderate consumption of alcohol, low intake of meat and meat derivatives, high consumption of vegetable, fruits, nuts and olive oil. Among vegetables, *Brassicaceae* family, are particularly beneficial as reported in several clinical trials or epidemiological studies (Wu et al., 2013; Jiang et al., 2014). Glucosinolates (GLs) are claimed to be the active components of cruciferous vegetables responsible for many beneficial effects assayed *in vitro*, and *in vivo* in human and in epidemiological studies (section 3.1.4.). Since 1992, the year of broccoli GL identification, more than 1000 studies have been conducted to determine the mechanisms and biological activities of sulforaphane (4-methylsulphinylbutyl isothiocyanate, ITC) and of its precursor, glucoraphanin (GRA, 4-methylsulphinylbutyl GL). When ingested sulforaphane has an half-life of ~2h, while little is known about the slow hydrolysis sustained by microbiota at intestinal level. Recent studies are trying to elucidate the metabolism of sulforaphane *in vivo* and it seems clear that the sulfoxide sulforaphane is in part converted in the liver, as well as in the gut microflora, in its thio-ether analogue erucin (4-methylthiobutyl ITC) (Clarke et al., 2011; Angelino et al., 2013). *Eruca sativa* Mill. seeds are the main holders of glucoerucin (GER, 4-methylthiobutyl GL), and its leaves contain also glucoraphanin, high levels of vitamin C, flavonoids, nitric oxide and phenols, which together contribute to the beneficial effects obtained by their sustained and controlled intake (**Figure 1**). The concentration of these molecules in food crops was in the past continuously lowered to moderate pungent taste and distinctive aroma which is not always appreciated by consumers. Non-food crops, such as oleaginous industrial crops of cruciferous family, can guarantee up to 100 $\mu\text{moles g}^{-1}$ of GLs at seed level, and this content could be even higher after meal defatting.

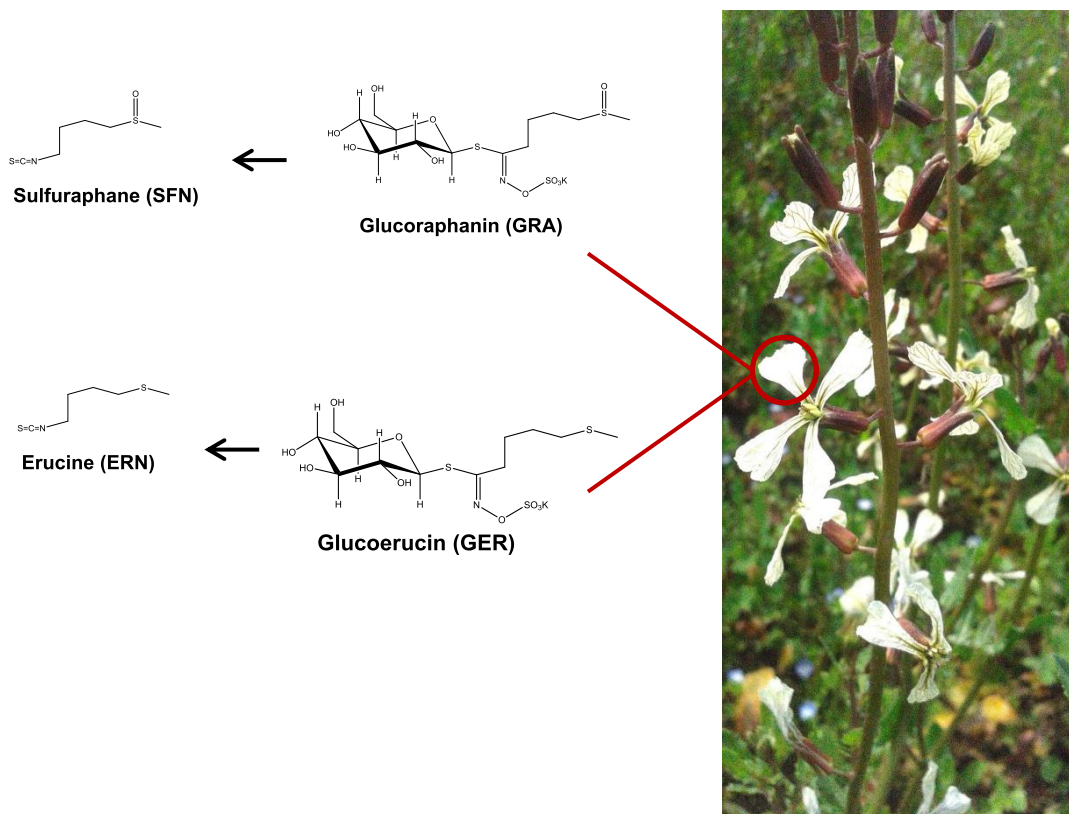


Figure 1. Main glucosinolates in *Eruca sativa* Mill. seeds and their isothiocyanate (Franco et al., 2016).

After a solvent-free oil extraction the residual defatted seed meal (DSM) can be considered not only a simple industrial waste but a true co-product of the oil production, particularly attractive for the food industry and for the production of functional foods with a high healthy value. Indeed *Brassicaceae* DSMs are particularly rich in protein, fibers, vitamins and GLs at concentrations not common for any other type of commercial horticultural product.

Four species of rocket seeds from the *Brassicaceae* collection of CREA-CIN (Lazzeri et al., 2013) were analyzed and one of them was selected to produce pressure defatted flours under controlled conditions in order to obtain a valid product for the enrichment of functional bakery products.

The design and production of glucoerucin and glucoraphanin rich bakery products was developed through the steps described as follows.

Starting material selection and optimization

The cultivar *Eruca sativa* Mill, Nemat, known for its use in biofumigation (Lazzeri et al., 2013), was selected and the main characteristics of the DSM, defatted under controlled conditions by pressing pilot extraction plant, were analyzed (Lazzeri et al., 2010; Lazzeri 2011). The obtained DSM showed a moisture content of 7.4%, 31% proteins and a 20.5% residual oil content, with 37% of erucic acid. DSM from *E.sativa* could be considered safe for food enrichment up to a maximum of 25% concentration, in order to not exceed the erucic acid European established limits (80/891/EEC, 1980).

Nemat DSM GL contents were determined by HPLC analysis of desulfo-GL following the ISO 9167-1 method with some minor modifications, using a Hewlett-Packard chromatograph 1100 equipped with a diode array detector, a ChromSep HPLC column SS (250 × 3.0 mm) and a ChromSep guard column Intersil 5 ODS-3 (Varian) (Franco et al., 2016). Sinigrin was used as external standard and the response factors of compounds were considered.

MYR activity was detected by the pH-stat technique as described by Finiguerra et al. (2001). One MYR unit (U) was defined as the amount of enzyme able to hydrolyze 1 μmol of sinigrin per minute at pH 6.5 and 37 °C.

Nemat DSM had a glucoraphanin (GRA) content of 0.44 μmoli g⁻¹ and glucoerucin (GER) of 89.8 μmoli g⁻¹ (**Figure 2**).

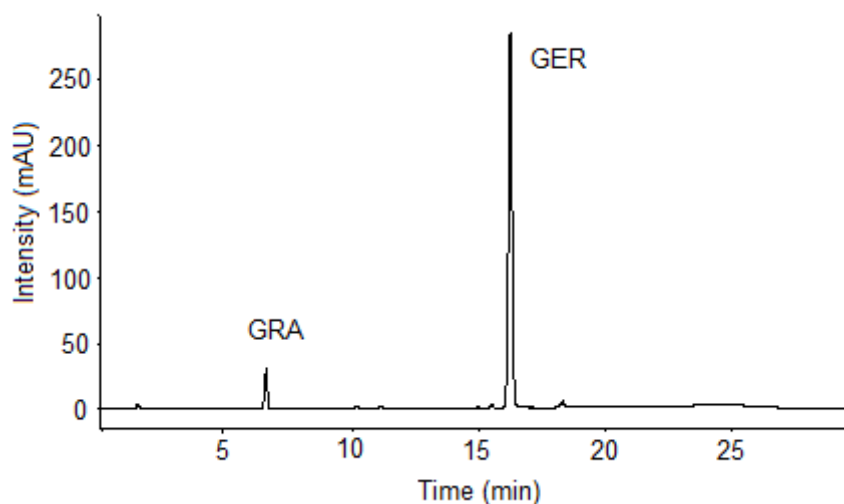


Figure 2. HPLC analysis of desulfo-GLs of Nemat *E. sativa* DSM.

Therefore peculiarities of the Nemat DSM are the high concentration in GLs and the presence of only GLs that are considered, at the moment, safe for food, with beneficial properties, as widely described in the literature (Dinkova-kostova and Kostov, 2012).

Recipes and products

Two rocket DSMs with different properties in term of MYR activity were prepared: one with partially active MYR (low pressure extracted DSM) and the other with a totally deactivated enzyme (high temperature pressure extracted DSM). A mild oil extraction by pressure in fact preserved 50% MYR activity (11 ± 2 U compared to the maximum MYR activity of 24 ± 3 U for *E. sativa* DSM, defatted by cold hexane). This allowed to try recipes with active MYR at the time of the mix (0-4) and recipes with MYR completely deactivated by heat treatments (5 and 6), preserving on the contrary intact GLs (**Table 1**). In deactivated DSM in fact the GER and GRA content were 74.6 and $0.47 \mu\text{moli g}^{-1}$ respectively. Recipes and final food products were produced in the R&D and QA of Colussi-Group (Perugia, Italy) by adding DSM to a standard cracker mixed with wheat flour baked at $200 \text{ }^\circ\text{C}$ in industrial plant (**Figure 3**). Further information on the recipes is commercially confidential and property of Colussi-Group.



Figure 3. Crackers products

Table 1. Cracker recipes with Nemat DSM added in different amounts, with active (0-4) or deactivated myrosinase (5 and 6).

Recipes	Supplements	Theoretical GLs ($\mu\text{moles}/100\text{ g}$)	MYR
0(b)	2,5 % Nemat DSM 1,3% Dehydrated broccoli	250	Yes
1(e)	2,5 % Nemat meal 1,3% Dehydrated broccoli	250	Yes
2(e)	2 % Nemat meal 1% Dehydrated broccoli	200	Yes
3(e)	1,5 % Nemat meal 1% Dehydrated broccoli	150	Yes
4(e)	1 % Nemat meal 1 % Dehydrated broccoli	110	Yes
5(b)	2,5 % Nemat meal 1,3% Dehydrated broccoli	210	No
6(e)	1 % Nemat meal 1 % Dehydrated broccoli	100	No

Where (b) stands for basic recipe, and (e) stands for enriched recipe (Colussi-Group).

Product quality control and troubleshooting

Isothiocyanate analysis. Free erucin concentration was determined in crackers by extraction in CH_2Cl_2 and GC analysis was performed by an Agilent 7820A GC system coupled with flame ionization detector (FID), and a Varian HP-5 capillary column (30 m length, 0.32 mm internal diameter, 0.25 μm film thickness). Instrument settings were as follows: injector and detector temperature were set at 200°C and 300°C respectively; the oven program started at 60°C and raised to 120°C at a rate of 10 °C min^{-1} ; the carrier gas (He) flow rate was 1 mL min^{-1} and the splitless injection mode was selected. Benzyl ITC (Sigma Aldrich) was used as internal standard and erucin content was calculated by using the previously determined response factor according to Brown et al. (1994).

Only recipe 1 presented appreciable amounts of free Erucin (33.3 $\mu\text{moles}/100\text{ g}$ product), but in very small quantities compared to the initial theoretical 250 μmoles , probably because of the instability of the molecule at the baking oven temperature (**Figure 4**).

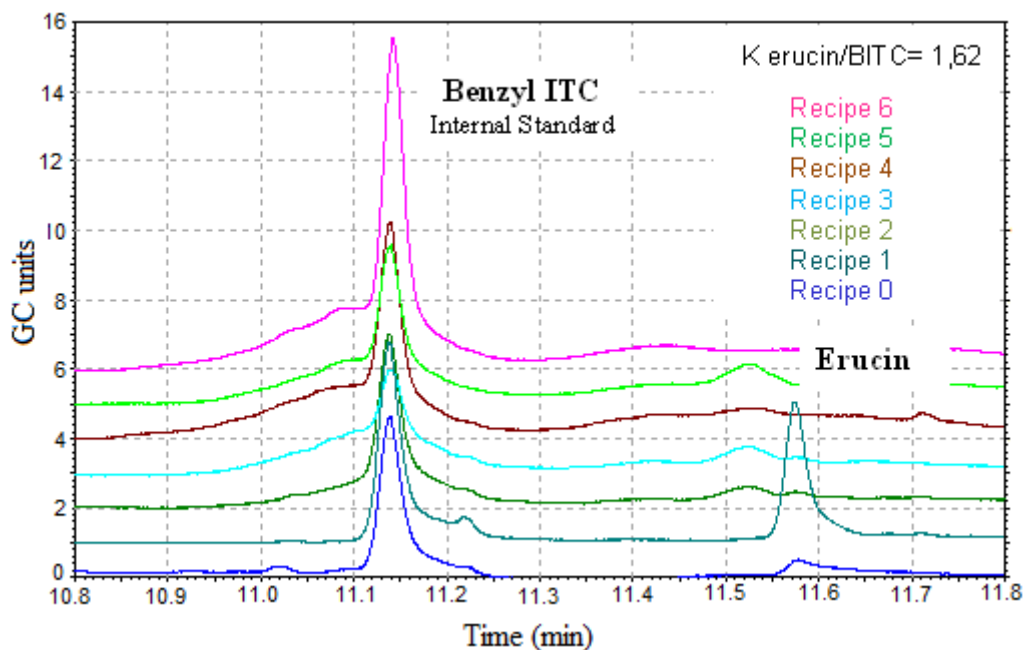


Figure 4. GC-FID analysis of the isothiocyanate erucin in crackers.

Intact GL analysis: the typical extraction of GLs with heated solvents was not sufficient for extracting GLs from bakery products. **Figure 5** shows results from three extraction procedures followed to achieve the best method, starting from recipe 6: **(A)** standard extraction in boiling 70% ethanol, according to ISO 9167-1 method (7 g in 20 mL of finely ground crackers); **(B)** standard extraction followed by a microwave assisted extraction step (4 g in 25 mL of finely ground crackers - 400 Watt, 80 ° C for 10 min, Green Chem Plus vessels, Mars5 CEM corporation System); **(C)** sonication (30 min, 40 kHz) in 70% boiling ethanol (4 g in 25 mL of finely ground crackers) and two microwave assisted extraction steps performed as before, in 70% ethanol.

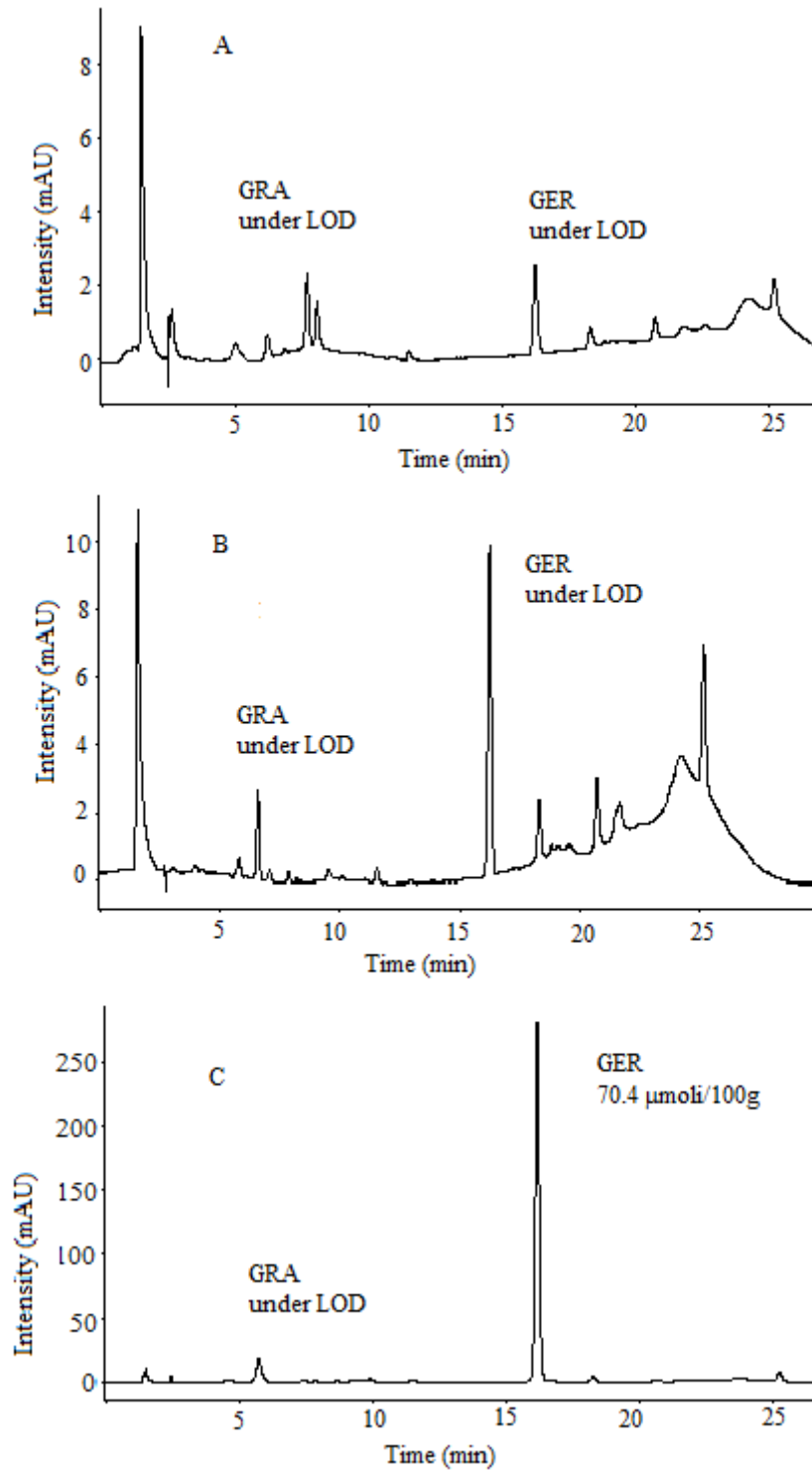


Figure 5. (A) HPLC determination of crackers GLs (recipe 6) according to the standard extraction ISO 9167-1 method, in boiling 70% ethanol; (B) GL extraction in boiling 70% ethanol followed by microwave assisted extraction step; (C) GL extraction via sonication step followed by two microwave extraction steps; LOD, limit of detection; GRA, glucoraphanin; GRE, glucoerucin.

The pre-sonication step followed by two microwave assisted extractions allowed a recovery of more than 96 % of total GLs, compared to the classical ISO 9167-1 method (13.5 %) and the single microwave assisted extraction (52.7 %).

Only products made with recipes containing deactivated MYR showed a high yield of GLs (70.4 μ moles/100g of GER for recipes 6), probably because active MYR determined GL hydrolysis during the dough preparation for the other recipes. It is worth noticing that the process of mixing and cooking caused the loss of only 5 % of GLs in the MYR deactivated recipe thus confirming the potential use of *Brassicaceae* meals in nutraceutical products.

Due to the low content of erucin ITC found in bakery product deriving from all designed recipes (**Table 1, Figure 4**), and to the low yield of GLs in products made with recipes containing MYR-activated DSM, MYR-deactivated DSM has been chosen to proceed with the development of the enriched bakery product. Such a DSM would be theoretically able to ensure an intake of GLs up to 70 μ mol/100 g of product, adding only 1% of *E. sativa* DSM to standard industrial recipes of crackers after baking at 180 °C. Such a conceived product may exert its beneficial effects only after the slow hydrolysis of GLs to ITC by intestinal microbioma. The addition of active MYR to the dough may be possible in the cooling phase of production using mustard seed meal (*Sinapis alba*), in order to increase GL hydrolysis rate. The determination of ITC and GLs during a simulated chewing process was also considered. DSM based cracker extracts obtained after stirring for 1 min with minimum quantity of artificial saliva (Perdigão et al., 1998) at 37 °C was re-suspended in boiling ethanol 70%, treated with microwaves (800W for 2 min) and analyzed by HPLC. The bakery products ensure a release of about 20 μ moles/100 g ITC during chewing, and the achievement of about 50 μ moles of GLs/100 g of crackers in the gastrointestinal tract for the stimulation of a slow hydrolysis by gut microbiota.

Conclusion

In this work an *Eruca sativa Mill.* cultivar was selected to produce pressure defatted oilseed meal under controlled conditions in order to obtain a valid product for the enrichment of functional bakery products. The development of an extraction method for the assessment of GLs in complex matrices permitted the quality control and improving of GL enriched bakery products. Developed methods for ITC and GL analysis in crackers made in fact possible to verify the production chain steps which led to the development of prototypes, interesting both for appeal and active substance concentration per portion.

The product developed giving the best results was able to release about 20 μ moles/100 grams of isothiocyanates during chewing, and to achieve in the stomach a concentration of intact GLs equal to about 50 μ moles/100 grams of product enough to stimulate a slow hydrolysis by intestinal microbioma (Angelino et al., 2013).

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4.2. Production of enzymatic protein hydrolysates from sunflower and rapeseed defatted seed meal and two potential applications

Oleaginous crops are widely used mainly for the oil fraction in non-food applications in green chemistry and for energy production, as in the biodiesel chain. Lately, there is a growing interest even in defatted seed meals (DSMs), as the main co-products of seed defatting. Usually DSM are used in low-value application as animal feed, but their steadily increase valorisation is becoming a fundamental opportunity with interesting economic and environmental perspectives (Lomascolo et al., 2012). The potential of the DSM carbohydrate fraction as a source of biofuels and bio-based materials has been extensively studied (Bozel and Petersen, 2010), while researches on proteins have received minor attention, despite their high economic value. Industrial oil extraction processes, though, may affect DSMs protein biological value, decreasing their solubility and bioavailability due to temperature/pressure/solvent denaturation (Lomascolo et al., 2012). To overcome this aspect, protein hydrolysates with enhanced functional properties have been studied for different agricultural applications (Ertani et al., 2009), as supplements in food and feed industry or as feedstock for the production of new bio-based chemicals (Gassman, 1983; Lammens et al., 2012). In this section the production of hydrolysates by mild enzymatic processes from sunflower (*Helianthus annuus L.*) and rapeseed (*Brassica napus L.*) DSM, and their potential application in two case studies are described and discussed. The first study explores the use of a sunflower meal hydrolysate (SMH) as plant biostimulant, while the second one investigates the use of sunflower and rapeseed meal hydrolysates (RMHs) as sources of protein in pet food, alternative to meat proteins. The different application fields also determined the choice of the hydrolysate production process. In the first case, in fact, the direct two-step extensive hydrolysis from sunflower DSM had the final aim to obtain a high percentage of free amino acids and low molecular weight peptides, while preserving other substances, which would make the product not suitable for feed application, but, on the contrary, could contribute to the biostimulant properties (Ordonez et al., 2007). In this perspective, some preliminary results were also outlined for the development of a biostimulant from DSM of *Brassicaceae*, which would include also bioactive glucosinolates. In the second case the hydrolysate production from sunflower and rapeseed DSMs was conducted through a one-step procedure, in order to obtain high percentage of medium-low molecular weight peptides and low amounts of free amino acids. In this case, a step of protein isolation before hydrolysis was needed, in order to obtain a product with a very high protein content and free of anti-nutritional compounds such as high fibres, phenols, chlorogenic acid (from sunflower DSM) or fitic acid and glucosinolates (from rapeseed DSM) (Parrado et al., 1991; Villanueva et al., 1999). Rapeseed and sunflower DSMs were produced by an industrial pressure-hexane oil extraction process, and provided by Itacol S.p.A

Castelfiorentino (Florence, Italy). Prior to analysis and hydrolysis trials DSMs were milled to a 0.5 mm size. DSM protein content were 31.1 % and 32.7 % (on dry matter basis) for sunflower and rapeseed, respectively. Results are summarized in the following sections (4.2.1; 4.2.2.).

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4.2.1. Production of an enzymatic protein hydrolysate from sunflower defatted seed meal for potential application as a plant biostimulant

Polypeptides, peptides and amino acids of animal and/or plant origin, obtained by protein hydrolysis, have been increasingly applied in agriculture, not only as a source of organic nitrogen, but also as a sustainable and renewable bio-based tool to improve soil quality and nitrogen acquisition efficiency of plants, contributing to reduce chemical fertilizer input (Maini, 2006; Schiavon et al., 2008). Amino acid containing products, together with microbial inoculants, humic substances, seaweed extracts, and other natural origin plant hormone-like compounds are in fact studied for their biostimulant properties (Calvo et al., 2014). Biostimulants were recently defined as substances and materials that, when applied to plants, seeds, or growing substrates in specific formulations, have the capacity to provide potential benefits to growth, development and/or stress response (Du Jardin, 2012). Different mechanisms of action were observed and studied as the improvement of soil microbial activity and soil enzymes and modulation of numerous plant physiology aspects, acting as positive growth regulators or metabolic enhancers. The biostimulant effect of hydrolysates, containing free amino acids and oligo-peptides, but also other bioactive non-protein components, has been demonstrated in many application studies, and related products have been successfully commercialized. Europe represented in 2012 the largest market for biostimulants which is a fast growing sector (+10% per year), deserving more and more research and development investments, especially in small and medium-size enterprises, SME (Maini, 2006; Ertani et al., 2009; Calvo et al., 2014). In Italy, vegetal extracts and hydrolysates rich in free amino acids (from alfalfa, *Fabaceae* and animal skin) fall within the biostimulant category according to the 75/2010 legislative decree. Accordingly, sunflower defatted seed meal (DSM), which contains about 30% of protein with a high quality amino acid profile, is an interesting starting material for the production of hydrolysates as bio-based supply of nitrogen and microelements with fertilizing properties, but also as a biostimulant.

Hydrolysate production

Hydrolysis protocol optimization. The use of enzymes from fungi and bacteria has become the most common choice for mild hydrolyses, avoiding the use of chemical processes, preserving the quality of free amino acids, and making sustainable the whole process. Preliminary trials aimed at finding the optimal hydrolysis conditions (temperature, pH, enzyme/substrate ratio, one or two step process) and the best starting material (DSM as such or subjected to component fractionation or protein isolation step). Hydrolysis was followed by the pH-stat technique (Adler-Nissen, 1986), where an automatic titrator maintained the pH at the desired value (Mettler-Toledo DL 50 Graphix).

The direct hydrolysis of DSM was carried out suspending it in water (10% w/v) and the combination of protein extraction and hydrolysis steps was chosen as the best method, enhancing the overall process efficiency: preliminary protein isolation step, in fact, determined a quantitative loss in terms of proteins and other bioactive compounds, without increasing the hydrolysis degree of the final product (data not shown). A two-step hydrolytic process by using Alcalase®, an endopeptidase from *Bacillus licheniformis*, and Flavourzyme®, an exopeptidase and endoprotease complex from *Aspergillus oryzae* (Sigma Aldrich), was performed in order to obtain a larger fraction of small peptides and amino acids. Optimal conditions were established as: i) preliminary incubation for 15 min at 50°C for maximum protein extraction; ii) a first step of hydrolysis with Alcalase enzyme, for 60 min, at 50°C, pH 8 and with an enzyme/protein ratio of at 0.2 AU/g; iii) a second step with Flavourzyme for 120 min, at 50°C, pH 7 and 50 LAPU/g as enzyme/protein ratio (Villanueva et al., 1999 for unit definition). The hydrolysis was stopped by adjusting to pH 6.0 with citric acid. This simplified approach made it possible to simultaneously extract and efficiently hydrolyse (hydrolysis degree DH 41% - Adler-Nissen, 1979) more than 75% of the protein content in DSM, minimizing time, costs and wastes of the process. An additional washing step of the residual meal could increase the protein yield to more than 90%, but it was not considered for the same economic considerations.

Sunflower meal hydrolysate characterization

Peptide molecular weight distribution. Peptide molecular weight distribution values were determined by SDS-PAGE, in 15 % polyacrylamide gels in Tris (Glycine SDS running buffer - 25 mM Tris-HCl pH 8.3, 192 mM Glycine, 0.1% SDS) and size exclusion chromatography using an AKTA fast protein liquid chromatography (FPLC) system equipped with a Superose 12 HR 10/30 column (Amersham Biosciences in 50 mM phosphate buffer pH 7, 0.15 mM NaCl, flow rate of 0.4 mL min⁻¹). Elution was monitored at 280 nm by a UV UCP-900 monitor (Amersham Biosciences). The electrophoretic profile clearly shows, at different incubation times, the hydrolysis of proteins with molecular weight higher than 30 kDa by Alcalase, while the action of Flavourzyme could not be highlighted as the free amino acids produced by the exopeptidase migrate with the front dye (**Figure 1 A**). **Figure 1 B** shows the gel filtration qualitative profiles of the final SMH and native DSM protein extract. It is clear how the hydrolysis led to a shift towards lower molecular size protein-peptide in SMH, if compared to the DSM extract profile, and to the appearance of low molecular weight compounds, probably new-formed amino acids and small peptides, but also other small compounds.

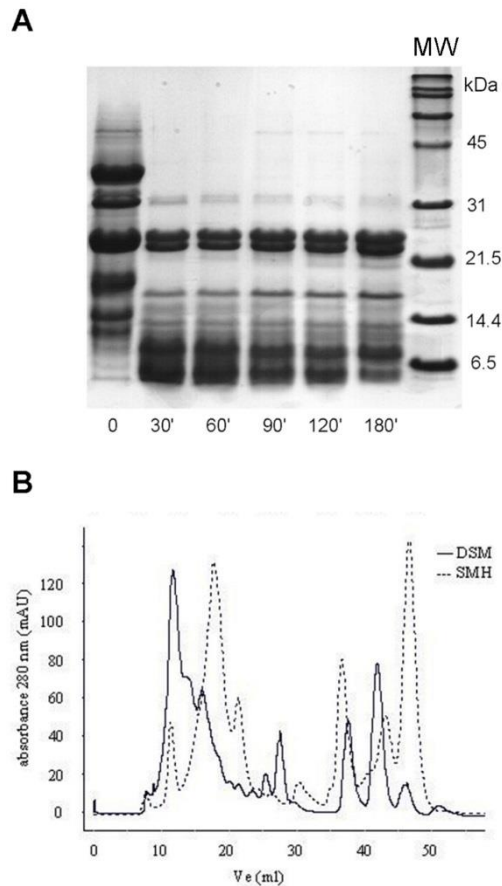


Figure 1. (A) SDS-PAGE of protein extract before the enzyme addition (time 0) and sunflower meal hydrolysates (SMHs) after different time intervals of hydrolysis (30, 60, 90, 120, 180 min): MW, molecular weight markers (kDa); Alcalase and Flavourzyme were added, at 0 and 60 minutes respectively. (B) Gel filtration chromatography of the native defatted sunflower meal (DSM) protein extract (—) and of the final SMH (---) on Superose 12 HR 10/30.

DSM and SMH chemical composition. The main chemical characteristics of DSM and freeze-dried SMH are presented in **Table 1** and show how the hydrolysis process permitted to concentrate the protein fraction and to eliminate the majority of the initial fibre content. The final freeze dried SMH had a 67.7% (w/w) protein content, corresponding to 10.8% of nitrogen, values comparable to the common commercial biostimulant products.

Other components deserve particular attention due to their possible involvement in the biostimulant activities, as plant growth promoters, auxin-like substances, soil microbial population enhancer or throughout different mechanisms of action:

- organic matter: the SMH had a good organic matter content, higher than other biostimulant (Nardi et al., 2002).

- indol-3-acetic acid (IAA): IAA was present in low amount, 0.8 mg kg⁻¹; (Ertani et al., 2013a);

- sugars: 16.9 % in SMH, mainly composed of glucose (44%) and fructose (36.5%). (Wang and Ruan, 2013).

- Amino acids: 16.4 % of the SMH and 24.3 % of the total protein content. (Cerdán et al., 2013).

- Micro and macronutrients were mostly preserved in the SMH.

Table 1. Chemical characterization of defatted sunflower meal (DSM) and sunflower meal hydrolysate (SMH). Analytical methods for moisture, oil, protein, ash, organic matter and carbon content are reported in De Nicola et al. (2013); fiber was analysed with FIWE analyser (Van Soest and McQueen, 1973), micro and macroelements, sugar and indol-3-acetic acid content were determined by the Research Institute for Agroindustry s.r.l. (Modena) (AOAC 958.01:1988; AOAC 922.02:1933; EPA 5050 1994; APAT CNR IRSA 2020 Man; Matsuda et al 2005). Data are reported as the means of three measures \pm standard deviation and are expressed on dry matter basis.

	DSM	SMH
Nitrogen %	5.0	10.8
Free amino acids (g Kg ⁻¹ , w/w)	ND ^a	164.4 \pm 6.7
Organic Matter %	93.1 \pm 0.4	75.8 \pm 0.0
Sugars %	2.4 \pm 0.3	16.9 \pm 1.5
Moisture %	7.5 \pm 0.6	9.5 \pm 0.3
Lipids %	0.8 \pm 0.1	ND ^a
Protein %	31.1 \pm 0.3	67.7 \pm 1.3
Fiber %	47.2 \pm 0.5	1.1 \pm 0.2
Ashes %	6.9 \pm 0.4	14.7 \pm 0.0
Carbon %	47.0 \pm 0.3	41.7 \pm 0.2
P %	1.1 \pm 0.1	0.33 \pm 0.01
Ca %	0.7 \pm 0.1	0.33 \pm 0.02
Na (mg Kg ⁻¹ , w/w)	83.0 \pm 3.1	232.9 \pm 8.4
K (mg Kg ⁻¹ , w/w)	8800.0 \pm 900.0	42199.0 \pm 4640.0
B (mg Kg ⁻¹ , w/w)	22.3 \pm 1.7	42.2 \pm 3.0
Co (mg Kg ⁻¹ , w/w)	0.3 \pm 0.03	0.18 \pm 0.02
Cu (mg Kg ⁻¹ , w/w)	25.3 \pm 1.2	43.2 \pm 2.0
Fe (mg Kg ⁻¹ , w/w)	298.4 \pm 5.5	50.1 \pm 0.9
Mg %	0.5 \pm 0.1	0.3 \pm 0.03
Mo (mg Kg ⁻¹ , w/w)	0.9 \pm 0.0	1.3 \pm 0.0
S (mg Kg ⁻¹ , w/w)	2809.0 \pm 0.0	5567.0 \pm 0.0
Zn (mg Kg ⁻¹ , w/w)	70.1 \pm 1.9	26.0 \pm 0.7
Indole-3-acetic acid (mg Kg ⁻¹ , w/w)	ND ^a	0.8 \pm 0.2

^aND : not determined

Amino acid composition. The free amino acid content of SMH, is reported in **Table 2** and was compared with the composition of two commercial protein hydrolysates, of different vegetal or animal source purchased from the market (VPH and APH).

SMH proved to contain a high and complete distribution of amino acids: twenty amino acids were detectable, at different concentration levels, in contrast to VPH and even more dramatically to APH. Only L-amino acids were present and tryptophan was also detected at 2.2%, thanks to the mild enzymatic hydrolysis condition which preserves them, differently from more aggressive chemical processes.

Amino acids have proved to have various beneficial effects on plants with different mechanisms of action: they act on nutrients and nitrate up-take, on the regulation of enzymes involved in N assimilation, resistance to biotic and abiotic stresses, secondary metabolism and plant growth promotion. The role of amino acid in plants was subject of different studies (Liu and Lee, 2012; Calvo et al., 2014) and there are evidences that plants can take up amino acids from the soil through specific transporters in roots (Hirner et al., 2006). Cerdán et al. (2013) found that foliar and root application of a vegetal hydrolysate, with 80% glutamic acid, enhanced ferrous root uptake and plant growth. Glutamine and arginine, the most abundant amino acids in SMH, were shown to have an effect on French bean growth, and secondary metabolism (Haroun et al., 2010). Forsum et al. (2008) showed that six amino acids individually promote plant growth on Arabidopsis, among which glutamine, asparagine and aspartic acid were most efficient. Some amino acids, like proline, protected plants from heavy metals and contributed to micronutrients uptake by means of chelating properties and exert antioxidant activities (Kaur and Asthir, 2015).

Nevertheless, a complex interaction between them, and among plant extrinsic and intrinsic factors could take part of the overall biostimulant effect (Forsum et al., 2008; Liu and Lee, 2012). Short peptides may also contribute to the improvement of micronutrient assimilation, and are involved in many aspects of plant growth, including defence response (Maini, 2006).

Table 2. Free amino acid composition (% w/w of total free amino acids) of sunflower meal hydrolysate (SMH), a commercial vegetal protein hydrolysate (VPH) and a commercial animal protein hydrolysate (APH). Amino acids were determined by reversed phase HPLC analysis and automated pre-column derivatization with o-phthalaldehyde-3-mercaptopropionic acid (OPA) and 9-fluorenylmethylchloroformate (FMOC) (Schuster, 1988; Henderson and Brooks, Application note 5990-4547EN 2010). A Hewlett-Packard Model series 1100 system, coupled with a diode array detector, and a Zorbax Extend-C18 column (4.6 x 150 mm, 3.5 μ m) was used.

	SMH	VPH	APH
	%	%	%
Aspartic acid	1.92	13.83	- ^a
Glutamic acid	2.67	14.06	- ^a
Asparagine	5.52	- ^a	- ^a
Serine	3.84	5.82	- ^a
Glutamine	14.38	- ^a	- ^a
Histidine	4.04	- ^a	- ^a
Glycine	2.05	3.37	39.50
Threonine	4.59	3.74	- ^a
Arginine	11.84	4.13	- ^a
Alanine	3.68	12.89	60.50
Tyrosine	4.96	3.91	- ^a
Cystine	1.34	- ^a	- ^a
Valine	5.97	6.50	- ^a
Methionine	3.48	- ^a	- ^a
Isoleucine	5.32	5.77	- ^a
Tryptophane	2.19	- ^a	
Phenylalanine	6.83	4.39	- ^a
Leucine	8.75	8.32	- ^a
Lysine	5.42	5.37	- ^a
Proline	1.21	7.90	- ^a

^a Under the limit of quantification.

Biostimulant assays

Biostimulant activity of SMH was demonstrated by two assays, the *in vitro* Audus test, and the *in vivo* pot test on *Zea mays*.

Audus test. The auxin-like activity of SMH was assessed by performing the Audus test (1972) on germination of *Lepidium sativum* L. (cress) and *Lactuca sativa* L. cv. Cosmic (lettuce), with some minor changes (Ertani et al., 2009) by using indol-3-acetic acid, IAA and gibberellic acid (Sigma Aldrich) respectively as reference standards. From results shown in **Table 3** it is clear that SMH application caused, on garden cress, a root shortening in a dose-dependent way (from 4 to 800 ppm), and a parallel improvement of root density together with the emergence of lateral branches and root hairs (**Figure 2 A and B**), similarly to what was expected from IAA treatment. It is well known, in fact, how auxins act as root growth inhibitor at high concentration, while they stimulate

roots at very low concentrations (about 1 part in 10^{11}) (Audus, 1972) and they are also the dominant regulators of lateral root development (Overvoorde, 2010).

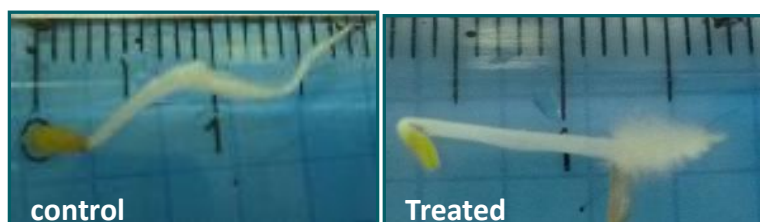
Anyway, the SMH content of IAA (**Table 1**) could not be the only responsible of the observed hormone like activity as it was present at too low concentration (0.8 mg kg^{-1} which means $3.2 \cdot 10^{-4}$ ppm in the SMH applied at 800 ppm) suggesting the contribution of other compounds. The amino acid L-tryptophan, for instance, is a precursor for auxin synthesis and an hormone-like effect on Arabidopsis root architecture was previously observed (Walch-Liu et al., 2006).

Conversely, no significant gibberellin-like activity was observed on epicotyls when SMH was applied.

Table 3: Auxin-like activity of Indolacetic acid (IAA) and sunflower meal hydrolysate (SMH) tested on garden cress roots. The test was performed by positioning sterilized seeds in filter paper in Petri dishes, wetted by 1.8 mL of solutions at different concentrations of IAA and SMH (Audus, 1972; Ertani et al. 2009). 1mM CaSO_4 solution was used as control. 50 measurements were performed per treatment. The values are expressed as percentages of root length decrease compared by Abbott's (1925) formula, to untreated control, measured after 72h, in the dark, at 25 °C. The arcsine transformed percentages were subjected to ANOVA and post hoc Fisher's LSD statistical tests ($P \leq 0.05$) were performed on arcsine transformed data by Statistica ® software (StatSoft Inc.USA). Significant differences between treatments are expressed by different letters.

Treatment	Concentration (ppm)	Root length decrease (%)
IAA	0.01	4.8 ef
	0.1	42.1 c
	1	75.5 b
	10	80.7 a
SMH	4	9.6 ef
	10	10.8 ef
	40	16.6 e
	400	30.3 d
	800	37.0 cd

A



B



Figure 2. (A) Garden cress roots in control (CaSO_4 solution) and treated samples (SMH 10 ppm in water). (B) Root morphology observed in bright field microscopy.

Pot test on Zea mays test. **Figure 3** shows the results of the sand bioassay performed on maize plant where the main root length was determined after SMH treatment, at different concentrations (from 0.9 to 900 ppm), in the presence or absence of nutrient Hoagland solution at 50% (Trinchera et al. 2010) with some modifications; Hoagland and Arnon, 1950).

SMH determined a 30 % maize root length increase over the control at low concentration in water solution (0.9 ppm) while at higher concentrations root length decreased (**Figure 3 A**). Biostimulant products, in fact, usually show their activity at low doses (1-10 ppm), thus differing from phyto-hormones, effective at even lower concentration (< 1 ppm), and fertilizer, active on the contrary at concentration higher than 100 ppm, depending on the real concentration of bioactive compounds in the product. Moreover, a decrease in root elongation was observed in control samples, compared to water treated plants, when plants had been treated with HS (nitrate concentration 7.5 mM), probably due to the consequent reduction of IAA levels in roots determined by high nitrate supply (up to 5mM) as observed by Tian et al. (2008). However, under SMH treatment, maize root partially restored elongation, probably mimicking the action of auxins (**Figure 3 B**). Root morphology also seemed to be influenced by hydrolysate application and again a more complex architecture was observed in the treated plant (**Figure 3 C**).

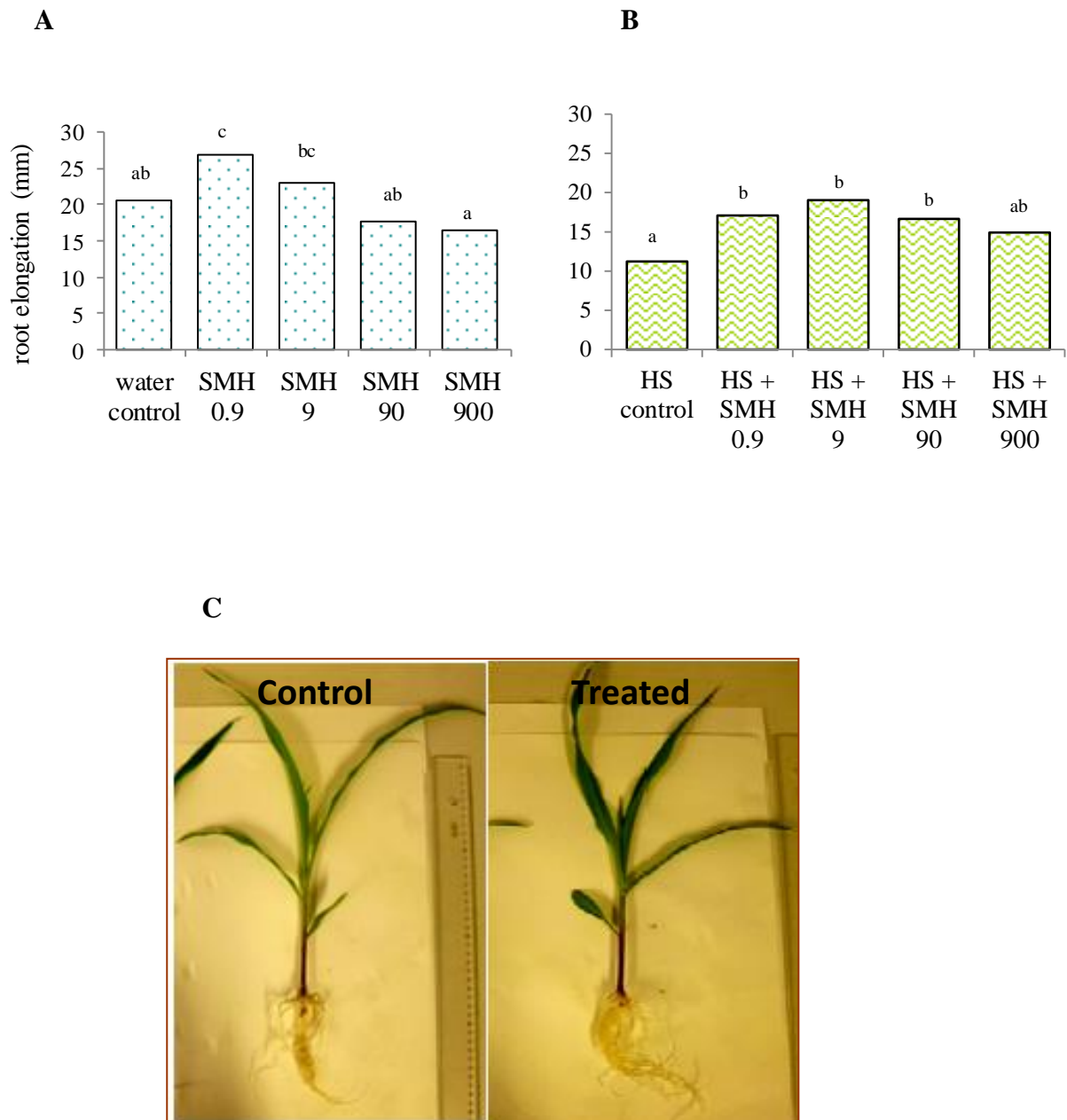


Figure 3. Root elongation of maize treated in pots with solutions (150 mL) at different concentrations of sunflower meal hydrolysate (SMH) in water (**A**) or in Hoagland solution at 50% (HS, **B**). (**C**) Control and treated (SMH 0.9 ppm) maize plant. Concentrations are expressed in ppm (mg of SMH on Kg of sand). Test were performed as described by Trinchera et al. (2010) on *Zea mays* (cv FAO 300) first germinated for 48h and after than two seedlings had been transferred in pot (6 cm of diameter) on sand. Plant were grown in growth chamber at 28°C with a photoperiod of 12/12 and relative humidity at 70% and evaluated for principal root length after two weeks. ANOVA and post-hoc Fisher's LSD statistical tests were performed. Significant differences between treatments (expressed by different letters) were determined by the LSD test ($P \leq 0.05$).

Other authors tested protein hydrolysates on maize seedlings hydroponically grown and evidenced how root and shoot increased their growth correlated with enhanced activity of enzymes involved in nitrate assimilation and carbon metabolism, partly ascribing those effects to the presence of indole-3-acetic acid, amino acids and small peptides (Ertani et al., 2013b). Furthermore the combined application of biostimulant products acting with different mechanisms of action could determine synergistic effects as observed for microbial-based and plant derived-protein hydrolysate biostimulants on lettuce (Rouphael, 2017).

Conclusions

In the last years biostimulants are object of common interest as alternative ways for increasing plant growth, limiting the extensive use of mineral fertilizers and going towards the public growing concern for human health conditions and environmental pollution.

The reported results confirm several previous experiences on protein hydrolysis, optimizing and improving some process steps. The possibility of a direct hydrolysis of the DSM valorises this co-product through an easier and lower-impact process, obtaining a product rich in free amino acids and other bioactive compounds. The final SMH showed an interesting effect on root growth, linked to an auxin-like activity, demonstrating the promising properties of the product as a biostimulant, even if further studies are needed to confirm this action. Amino acids, humic substances, microelements and sugars in SMH could coordinate, as and with, auxin-like compounds in complex signaling cross-talks promoting plant growth, favouring plant transplanting and final crop yield.

Finally, hydrolysates from a variety of plant residues have been studied and compared with animal origin hydrolysates, not only for their different and often better efficacy (Cerdán et al., 2013), but also being considered biologically safer and are easily accepted by consumers. In fact, even if Corte et al. (2014) recently showed the absence of toxic and genotoxic effects, on different bioassay systems, of some hydrolysates derived from the leather industry, the EU banned the application of such animal protein hydrolysates on the edible parts of organic crops, through the Commission Implementing Regulation (EU) n° 354/2014 with regard to organic production, labelling and control (Colla et al., 2014). In this view, bio-based products of vegetal origin play an important role, also considering the debate on the use of animal hydrolysate in organic farming.

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4.2.2.1. Exploring the use of other defatted seed meals for plant biostimulant production

Preliminary evaluation of isothiocyanates and Brassicaceae extract

The study of different defatted seed meals (DSMs) characterized by interesting biostimulant properties not only limited to protein content but also for the presence of bioactive secondary metabolites is currently ongoing. Among them the *Brassicaceae* family deserves particular attention for the glucosinolate (GL) content, some of which structurally related to the recognized plant hormone indol-3-acetic acid. The effect on plants of GLs and derived products have not been deeply studied yet. Some results were obtained in *in vitro* assays by treating maize seedlings with *Brassica napus L., var. oleifera* extracts: root length increased and radicle morphology modifications were registered at 0.1-1 ppm (w/v) suggesting an evidence for the biostimulant activity of the extract (Rivera et al., 2010).

Some experiments were therefore conducted in order to investigate the possibility of obtaining a new biostimulant product from *Brassicaceae* DSM, enriched in GLs, by testing a GL degradation product and a DSM extract in *in vitro* bioassays. A preliminary screening of different isothiocyanates (ITCs) (not shown) brought to the selection of the gluconasturtiin ITC (phenethyl ITC), largely known for its chemo-protective and anti-microbial properties (Conaway et al., 2005; Kim and Lee, 2009). This ITC is formed from the corresponding GL found in *Barbarea verna Mill Asch.*, a species characterised by winter hardiness, relatively large and high yield seeds, with a good potential to become a valuable oilseed crop (Andersson et al., 1999).

Experimental. The effect of pure ITC, hydrolysis product of the *Brassicaceae* secondary metabolite gluconasturtiin, and of the *Barbarea verna* DSM extract, containing the same molecule, was tested on *Lepidium sativum* seedling roots by the Audus test performed as described before (Audus, 1972; Ertani et al. 2009). ITC was formed *in situ* by purified myrosinase (MYR) enzyme and GL, in 25mM 2-(N-morpholino)ethanesulfonic acid, MES, buffer, pH 6.5 (**Figure 1**) and its concentration (ppm) was expressed as starting GL concentration, as pure molecule or as component of *B.verna* DSM extract (hydrolysis conversion GL-ITC = 100%).

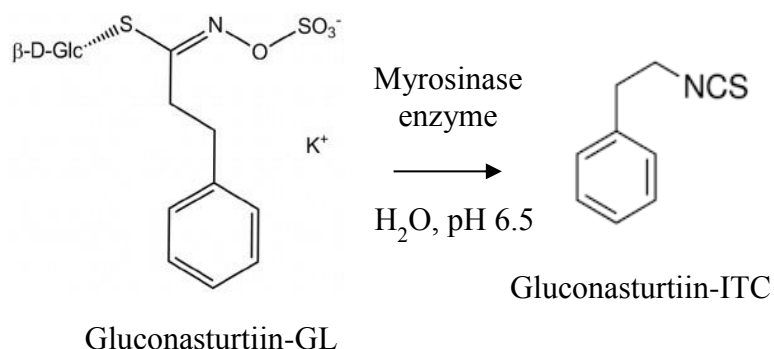


Figure 1. Myrosinase catalyzed hydrolysis of gluconasturtiin (phenethyl) glucosinolate into the corresponding isothiocyanate.

B. verna extract was prepared in the same buffer (1:10 w/v). 1.8 mL of solution containing the ITC or extract was added, after centrifugation, to the filter paper inserted in the Petri dish (9 cm diameter). After 72h at 20°C, in the dark, the garden cress root length was measured. 25 mM MES pH 6.5 was used as control. Experiments were replicated three times with 10 seeds each.

Myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147), was isolated from *Sinapis alba* seeds as reported in Pessina et al. (1990). MYR activity was detected by the pH-stat technique as described by Finiguerra et al. (2001). The enzyme solution was stored at 4 °C in sterile distilled H₂O until use and its activity (36 U/mL) was tested before each use. One MYR unit (U) was defined as the amount of enzyme able to hydrolyze 1 μmol of sinigrin per minute at pH 6.5 and 37 °C.

Gluconasturtiin GL was extracted and purified according to Barillari et al. (2005) with some minor modifications. The GL purity was improved by gel-filtration performed using a XK 16/60 column packed with Sephadex G10 chromatography media (Amersham Biosciences), connected to an FPLC System (Pharmacia). The purity of GL assessed by HPLC analysis of desulfo-GL by following the ISO 9167-1 method with some minor modifications, by using a Hewlett-Packard chromatograph 1100 equipped with a diode array detector, a ChromSep HPLC column SS (250 × 3.0 mm) and a ChromSep guard column Intersil 5 ODS-3 (Varian) (Franco et al., 2016).

Isothiocyanate production from pure GL or from DSM, and its identification was checked by ethyl acetate extraction (1:1 v/v) and GC-MS analysis: Bruker GC 451 gas chromatograph equipped with a HP-5 fused silica capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness, J&W Scientific Inc, Folsom, CA) connected to a quadrupole mass detector (Bruker Scion

SQ Premium, Bruker Daltonics, Macerata, Italy) was used. The oven temperature was set at 60 °C, and maintained for 4 min, after that it was programmed to rise from 60 to 220 °C at 10 °C min⁻¹, and finally held at 220 °C for 4 min. The transfer line was maintained at 280 °C and the ion source at 220 °C. Split injection (1:20) was applied and the carrier gas flow (helium) was 1 mL min⁻¹. The mass spectrometer was operated in electron impact mode at 70 eV, scanning the range of 10-250 *m/z*, in a full scan acquisition mode. Mass spectra were identified by matching the recorded mass spectra with the NIST/EPA/NIH Mass Spectral Database (NIST 11, Gaithersburg, MD), or compared with those of ethyl acetate solutions of authentic standards.

Results. The results showed an inhibition by gluconasturtiin-ITC on garden cress root elongation respect to the control at doses higher than 31 ppm, while an enhancing effect was observed at the lowest doses (**Figure 2**). This trend resembles the typical behavior of auxin-like compounds, which act as root growth inhibitor at high concentration, while they stimulate roots at very low concentrations (about 1 part in 10¹¹) (Audus, 1972). Anyway lower concentration (≤ 4.6 ppm) should probably also be tested in order to observe a more pronounced biostimulant effect. A similar trend was observed also with the *B.verna* extract, even if much less pronounced.

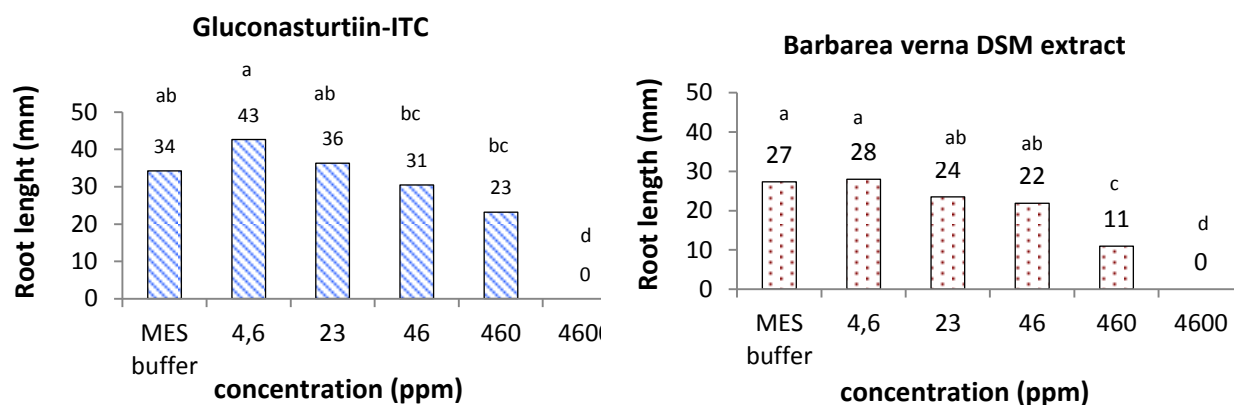


Figure 2. Auxin-like activity of gluconasturtiin derived isothiocyanate and *Barbarea verna* defatted seed meal extract, tested on garden cress roots (Audus test). ANOVA and post-hoc Fisher's LSD statistical tests were performed. Significant differences among treatments (expressed by different letters) were determined by the LSD test ($P \leq 0.05$).

These preliminary results showed an interesting perspective on the production of a biostimulant product from *Brassicaceae* DSMs, with the aim of preserving the GL together with

other possible bioactive molecules. The product could in fact contain GLs, but also amino acids and peptides that can be produced by hydrolysis of the DSM protein fraction, thus combining the two possible biostimulant effects. Further researches on the synergic interaction between amino acids and peptides with GLs, but also phenolics or phyto-hormones, should deserve more attention, even more considering the wide availability of bioactive compounds from other residual DSMs. In fact, among residual biomasses derived from industrial oil production, other DSMs (*Camelina sativa L. Crantz*, *Linum usitatissimum L.*, *Carthamus tinctorium L.*, *Crambe abyssinica R.E.Fr.*), interesting for their proteins and bioactive compound content which could play a role in the biostimulant activity, are under investigation.

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4.2.2. Production of an enzymatic protein hydrolysate from defatted sunflower and rapeseed seed meal as alternative protein sources for pet food

Sunflower and rapeseed defatted seed meal (DSM) are the main co-products of the vegetal industrial oil production chain, and may constitute an optimal source of proteins for human and animal nutrition as they contain between the 30 and 45% of protein. Protein composition of both DSMs is represented by a low level of low molecular mass (14 kDa) as albumin (20-30%), and a high level of higher molecular mass (300 kDa) as globulin (55-70%), named napin and cruciferin respectively, in rapeseed (Ordonez et al., 2007; Wanasundara et al., 2016). The two DSM have a high quality and balanced amino acid profile, except for a deficiency in lysine in sunflower DSM, compared to most legumes and animal protein sources. Rapeseed in particular satisfies the FAO requirements and is rich in sulfur containing amino acids and lysine too.

Today the mostly utilized vegetal proteins as food supplements are those from soy, barley and maize, but different research studies have considered also hydrolysate/extracts from sunflower and rapeseed DSMs (Villanueva et al., 1999; Yoshie-Stark et al., 2006; Chabanon et al., 2007). Rapeseed proteins were studied as a substitute or supplement of animal or vegetal proteins in processed food, improving their technological and nutritive values (Mansour et al., 1996; Cumby et al., 2008).

Anyway several non-protein chemical constituents of the seed, as fibre, phenols, or glucosinolates, could be associated to proteins and could alter their nutritional value and functional properties. Furthermore napin, mustard (*B. juncea* and *S. alba*) and derived products are recognised as human gastro-intestinal allergens; proteins capable of eliciting immunogenic response could represent a problem also in sunflower and rapeseed DSM-derived food (Monsalve et al., 2001, Wanasundara et al., 2016).

The production of protein isolates and/or protein hydrolysates from DSMs could overcome those hindrances, improve the quality and stability of hydrolysate-based nutritional formulas and represent an alternative source of protein for the food industry and especially for pet food. This need arises from the growing demand of products, in particular for dogs and cats suffering from adverse food reactions (AFRs) (Kang et al., 2014; Willis-Mahn et al., 2014; Suto et al., 2015). Hydrolysed proteins are less allergenic and more digestible (Sá et al., 2013), but the protein hydrolysates currently used for this purpose mainly derive from animal by-products and non-EU seeds as soybeans, implying an expensive economic and environmental effort. Trying to overcome this problem, rapeseed and sunflower DSM-derived hydrolysates (RPH and SPH) were investigated for their possible application in this context.

Hydrolysate production

Protein isolate preparation: protein isolates were obtained from DSM by alkaline extraction (KOH 2 M, pH 10.5, 1:10 w/v, 1 hour in agitation) and isoelectric precipitation with citric acid (at pH 4.3 for sunflower and pH 5.0 for rapeseed proteins) as described by Villanueva et al. (1999) and Vioque et al. (1999). However, differently from the cited works, the sedimentation/flotation method and aqueous ethanol washing steps, performed to eliminate fibres, sugars and phenolics and obtain a higher protein purity, were skipped not being achieved a real protein purification improvement (data not shown), especially considering the overall cost/benefit ratio on a large scale production. No sodium sulphite was added, as antioxidant, to the alkaline extraction solution, as it did not influenced the final product browning, possibly due to the polyphenol oxidation. After protein precipitation, extracts were separated by centrifugation. Extraction and precipitation of sunflower proteins were performed at room temperature with yields of 84% and 48% respectively. The final protein isolate had a high protein content (92.2 % w/w on dry weight). For rapeseed a lower extraction yield was obtained, 58%, when temperature of 40 °C was applied, while higher or lower temperatures did not improve the process yield. The final protein isolate had a 73.4 % protein content. The lower protein content respect to sunflower DSM could have been caused by co-precipitation of other non-protein components. Anyway extraction yield could be increased to more than 95 % with a second extraction, but a one-step procedure was nevertheless chosen for further productions, minimizing time and costs.

Hydrolysis protocol optimization. A mild enzymatic one-step hydrolysis of DSM protein isolates previously prepared was performed by using the food-grade proteases Alcalase® (endopeptidase from *Bacillus licheniformis*, Sigma-Aldrich), to obtain a product rich in small peptides, that could be more easily absorbed by the animal intestine than the original proteins (Parrado et al., 1991, 1993). Protein isolates were suspended in distilled water (12.5 %, w/v), and the enzyme was added at the concentration of 0.2 AU g⁻¹ (Alcalase-meal protein ratio). The process was followed by the pH-stat technique (Mettler Toledo DL 50 Graphix), for 60 min, at pH 8 and 50°C and stopped by adjusting the pH to 6.0 with citric acid. The final sunflower and rapeseed protein hydrolysates (SPH and RPH) were characterised by a protein content of 83.2 and 70.2 %, respectively (**Table 1**). Other components (ashes) are probably derived by the reagents used for pH control during the hydrolysis process (citric acid and KOH). Nevertheless, protein purity could be improved by using volatile reagents or by reducing their addition (just using them for setting the initial pH), and conversely exploiting the buffer capacity of the isolated proteins to maintain pH during hydrolysis (results not shown). Phenolics and other non-protein compounds (fibre, lipids, soluble sugars, glucosinolates) were mostly eliminated by the preliminary precipitation process as

reported by Villanueva et al. (1999), and the final hydrolysates showed good characteristics to be tested as pet food protein integrators. It has to be noted that the starting rapeseed DSM used in this trials derived from a ‘double-low’ cultivar and had a low content of GLs known for their goitrogenic properties (mostly progoitrin, *R*-2-hydroxy-3-butenyl-GL, 4.9 $\mu\text{mol g}^{-1}$). The complete absence of those GLs in the hydrolysates was confirmed by standard GL analysis (section 4.1).

Table 1. Chemical composition of sunflower and rapeseed defatted seed meal (DSM) and hydrolysates. Methods are reported in section 4.2.1.

	Sunflower DSM	Rapeseed DSM	Sunflower hydrolysate	Rapeseed hydrolysate
Protein %	31.1±0.3	32.7±0.1	83.2±1.8	70.2±1.2
Ashes %	6.7±0.1	7.0±0.1	14.4±0.8	13.8±1.2
Moisture %	8.3±0.6	9.5±0.5	9.8±0.3	9.9±0.3

Hydrolysate characterisation

The composition of the two hydrolysates SPH and RPH was determined by SDS-PAGE and size exclusion chromatography as described in the previous section (4.2.1.). The SDS-PAGE analysis showed the presence of peptides with a molecular weight lower than 25 KDa and 14 KDa for SPH (**Figure 1**) and RPH (**Figure 2**), respectively. The gel filtration chromatography profile clearly indicated the shift towards lower molecular size protein-peptide in SPH and RPH, if compared to the protein isolates, and showed the presence of new-formed low molecular weight compounds, represented by peaks with elution volume higher than 15 ml. This could be probably due to the presence of small peptides (as showed by SDS-PAGE) and a low percentage of amino acids (**Figure 1 and 2**). The use of Alcalase alone, in fact, permitted a mild protein hydrolysis (hydrolysis degree DH 18%, Adler-Nissen, 1979) and the SPH and RPH hydrolysate content of total free amino acids determined by HPLC analysis following OPA-FMOC derivatization (see section 4.2.1.) was limited to 0.6-1.0% w/w.

Figure 1: Gel filtration chromatography of the sunflower protein isolate (—) and the final sunflower protein hydrolysate (---) on Superose 12 HR 10/30. **Inset:** SDS-PAGE of sunflower protein hydrolysates (SPH); MW, molecular weight markers (kDa).

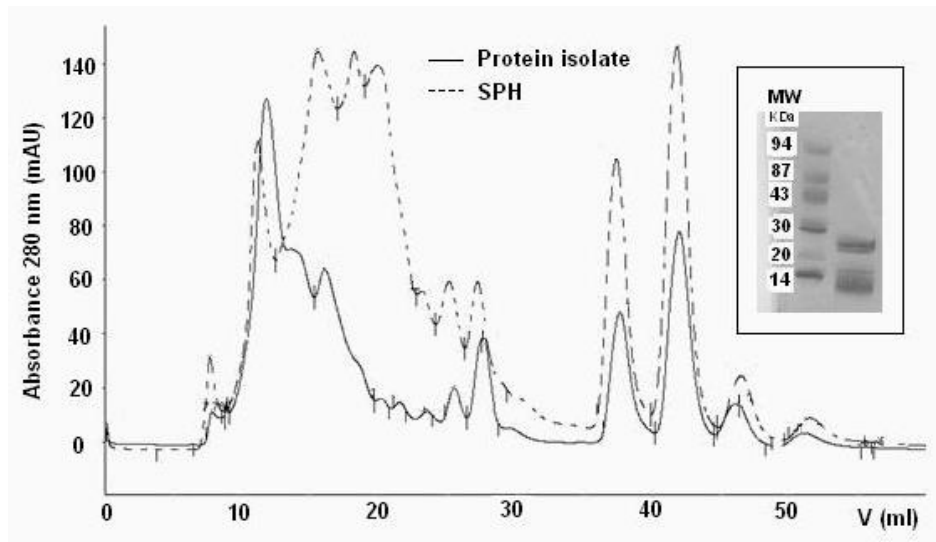
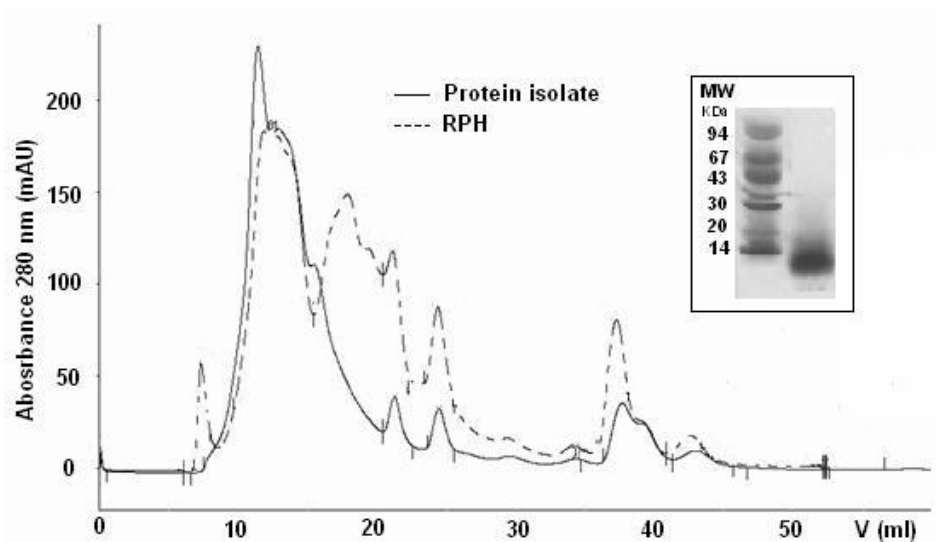


Figure 2: Gel filtration chromatography of the rapeseed protein isolate (—) and the final rapeseed protein hydrolysate (---) on Superose 12 HR 10/30. **Inset:** SDS-PAGE of rapeseed protein hydrolysates (RPH); MW, molecular weight markers (kDa).



In vitro digestibility test and in vivo safety evaluation

The digestibility, sub-acute toxicity and metabolic effects of diets containing DSMs or RPH and SPH have been evaluated in a murine animal model, at the Department of Veterinary Medical Sciences, and the Department of Pharmacy and Biotechnology, Alma Mater Studiorum-University of Bologna (Canistro et al., 2017), as a prerequisite for determining the suitability of these foodstuffs to be potentially added to pet diets.

The digestibility of a mix of commercial cereals for dogs and DSMs or hydrolysates (5:4.5 g) was tested *in vitro*, simulating the enzymatic digestion that takes place in the stomach and in the

small intestine of dogs. Both hydrolysates showed higher *in vitro* digestibility than the respective DSMs, presumably as a consequence of the hydrolysis process that they had undergone, and the absence of fibre.

For *in vivo* experiments mice Swiss Albino CD1 were fed for 28 consecutive days with diets containing 10% of RPH or SPH, with the aim of showing up both sub-acute adverse toxicological effects in major vital systems and the modulation of drug metabolism enzymes.

Animal body weight and food intake, general signs and symptoms, blood biochemistry parameters, phase I and II enzymatic activities were determined. The results showed that RPH and SPH were well tolerated and there was no evidence of toxicological effects. Body and organ weights, biochemical blood measurements of treated male mice were comparable to controls. Food intake was regular in suggesting a good palatability of the hydrolysates. Not relevant perturbations of the principal hepatic and renal drug metabolism enzymes were observed (Canistro et al., 2017).

Conclusions

The market of industrial pet food, especially for those with a suspect or a confirmed diagnosis of adverse food reactions (AFRs), is currently growing. More recently, hydrolysed veterinary diets have been proposed for the diagnosis of canine AFRs (Gaschen and Merchant, 2011). During hydrolysis, protein sources, are enzymatically broken down to low molecular weight peptides, changing and reducing the allergenic properties of the molecules (Boumans, 2001; Cave and Guilford, 2004). The employment of rapeseed and sunflower DSM easily useful and broadly available, could represent a valid opportunity for solving this problem with a cost reduction. A first protein isolation step from the DSM was necessary in order to obtain a product free of anti-nutritional compounds, and a high protein content. The obtained RPH and SPH were finally tested for a safety assessment, in the sub-acute toxicity study, and the observations in this study imply that mice well tolerated the hydrolysates when added to their diet. Moreover, RPH and SPH did not alter the hepatic and renal drug metabolizing enzymes.

In conclusion, the present findings encourage further exploration of the protein hydrolysates from sunflower and rapeseed DSM as alternative protein sources in several applications, such as food for pets suffering from adverse food reactions.

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GENERAL CONCLUSION

The results reported in this thesis showed different possibilities of high value product diversification starting from defatted seed meal (DSM). This is a co-product derived from industrial oil extraction processes from seeds of oleaginous crops of great economic importance, such as *Brassicaceae* (Rapeseed, Carinata et al.) and of *Asteraceae* (Sunflower et al). Bioactive molecules, as glucosinolate derived products, and proteins, were employed for plant management and defense in several agricultural systems or as food or feed supplements, with interesting and promising results.

In particular biofumigation with allyl-isothiocyanate (AITC), produced from formulated brassicas DSMs, from *Brassica carinata* A. Braun or *Brassica nigra* L., previously set up and characterized, permitted to obtain good results on the shelf-life extension of fruit in the postharvest phase. In strawberries in fact the treatment with AITC vapors (0.1 mg L⁻¹, 4 h) significantly contained the incidence of postharvest fungus *Botrytis cinerea* by over 45%, up to 91.5%, without affecting the overall nutritional properties of the fruit. Low AITC residues were detected in fruit after a short storage (<1m Kg⁻¹ of fruit). In kiwifruit, a similar treatment (0.15 mg L⁻¹, 5 h) preserved quality and enhanced the nutraceutical properties in terms of increased antiradical capacity and phytochemical content, without leaving any detectable AITC residues on fruit after long storage in controlled atmosphere. AITC influence on ethylene, ascorbic acid, phenylpropanoid biosynthesis was also depicted at gene expression level in kiwifruit. Furthermore the GSH cycle involvement in AITC mechanism of action/detoxification was confirmed in both fruits.

Among other DSM application studies *Brassicaceae* were also used for the development of functional bakery products enriched in bioactive molecules. A prototype of crackers with interesting properties both for appeal and active substance concentration was set up starting from *Eruca sativa* Mill. DSM.

Finally, different components of other DSMs were explored: the sunflower (*Helianthus annuus* L.) and rapeseed (*Brassica napus* L.) DSMs, largely produced oil extraction co-products, characterized by an interesting protein content, were employed for the production of protein hydrolysates through mild enzymatic processes.

The obtained sunflower DSM hydrolysate, rich in free amino acids and other potentially biological active molecules, showed, in *in vitro* and *in vivo* assays, promising properties for plant biostimulation with significant effects on plant roots, linked to an auxin-like activity. The product could represent, particularly in organic farming, a valuable biostimulant vegetal alternative to the

animal hydrolysate, which use on the edible parts of organic crops was recently banned by EU (n° 354/2014). In this context the potentiality of Brassica DSMs as biostimulant source was also preliminarily evaluated, obtaining results with *Barbarea verna Mill. Asch.* isothiocyanate.

Again, sunflower DSM, but also rapeseed DSM-derived protein hydrolysates, were also produced for the development of pet food supplements that were tested in mice, obtaining satisfactory results in term of digestibility, toxicity, safety, and metabolic effects.

In conclusion, the presented approach aimed at valorizing the biomasses, considered as residues of low value, and change their role from by-products of the oil extraction process to co-products, with high added value for applications in different agro-food sectors.

The attention to the entire biomass and the full utilization of crop raw materials to produce a wide range of different ‘green’ materials based on DSMs, represents today a fundamental bet for the development of an economic and environmental valuable, sustainable and innovative biorefinery chain.

Furthermore the reliance on innovative bio-based products as an alternative to conventional chemical sources, such as the examples reported in this study, is today more and more necessary for the human and environmental safety and well fit with the EC Regulation (EC) N° 1907/2006 concerning the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) which has the aim of regularizing the entire chemical sector and of limiting its environmental impact. This strategy is therefore strongly encouraged by the European union for the development of a new circular economy prospective, in addition to be always more frequently requested by consumers.

PUBLICATIONS ARISING FROM THE PHD PROJECT

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Others:

- The PhD student Luisa Ugolini was **Guest Editor** for the *Industrial Crops and Products* journal, volume 75 part A (**2015**), of the special number entitle 'Biodiesel chain', which gather publications arising from the VALSO project, (technology integrated system for the valorization of the by-products derived from the biodiesel production chain), ended in 2014, in which she performed a part of the research work within the PhD program.

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