Cold atmospheric pressure plasma treatment of food matrices: Tailored modification of product properties along value-added chains of plant and animal related products

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"God, grant me the serenity to accept the things I cannot change,
Courage to change the things I can,
And wisdom to know the difference."

Reinhold Niebuhr

Dedicated to my beloved grandfather – my guide, guardian, and companion…

Hans-Joachim Thieme († March 15th, 2005)

Preface

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5. Bußler, S., Steins, V., Ehlbeck, J., Schlüter, O. (2015): *Impact of thermal treatment versus cold atmospheric plasma processing on the techno-functional protein properties from* Pisum sativum *'Salamanca'.* Journal of Food Engineering. 167 (Part B): 166-174.
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Abstract

The application of cold atmospheric pressure plasma (CAPP) was suggested as an innovative nonthermal technology for inactivating undesirable microorganisms on the surface of heat-sensitive food products. Moreover, CAPP may offer a promising approach for the tailored modification of product properties along value-added chains of plant and animal related products. Therefore, this thesis puts emphasis on providing evidence for the possible utilization of plasma-induced surface and ingredient interactions as a tool for the selective modification of secondary metabolite profiles in plants and techno-functionality of flours and proteins from peas. Further, the knowledge gained on plasma assisted modification of plant-based materials was transferred to animal-based materials from edible insects taking additionally into account microbial decontamination as another key issue in insect processing. The thesis imparts the detailed characterization of selected raw materials, the identification of proper CAPP setups and corresponding product-specific process parameters by performing process accompanying monitoring of plasma characteristics required to achieve desired modifications. Detailed investigations of the plasma-induced effects were conducted following a top-down approach by using suitable analytical methods providing insights into possible underlying mechanisms from macroscopic to molecular level. The macroscopic level of analysis included quality (color/texture), compositional (protein/fat/dry matter contents) and microbial (surface/overall total viable counts) methods, followed by determining techno-functional (water/fat binding/emulsification) and protein (solubility/water/fat binding/emulsification) properties, as well as protein structure (surface hydrophobicity/fluorescence properties/CD spectroscopy) on the microscopic and structural level, down to analysis of the protein composition (SDS-PAGE/tryptophan content/amino acid composition) on the molecular level. The results provide a scientific basis regarding the targeted use of the CAPP technology for functionalization and modification of high-protein food components and could therefore contribute to the bio-economic and resource efficient production of dry high-value protein products, as protein functionality plays a key role in improving existing products, developing new products, and utilizing alternative protein sources as new ingredients. Consequently, feedback on the applicability of CAPP for tested raw materials was derived by evaluating the effectiveness of the treatment regarding the desired process goal, and by identifying product-specific characteristics allowing transferability of the CAPP process with the long-term goal of combining plasma treatments with existing unit operations in established product-specific process lines. Initial approaches regarding the CAPP treatment of complex food matrices by using a plasma device, which is promising for the application in industrial scale, aimed at the development of innovative process combinations with focus on “plasma-drying” as a prospective future unit operation, which may contribute to reducing the expected costs of CAPP treatments. The potential of CAPP processing to become a routine tool for the food industry in the coming years is also reflected throughout all parts of this work, and thus, it provides a substantial contribution to promoting the successful admission of the CAPP technology in the food sector.

Zusammenfassung

Kalte Atmosphärendruckplasmen (KADP) gelten als innovative nichtthermische Technologien zur Inaktivierung unerwünschter Mikroorganismen auf Oberflächen hitzeempfindlicher Lebensmittel. Darüber hinaus bietet die KADP-Technologie ein vielversprechendes Konzept zur gezielten Modifikation von Produkteigenschaften entlang Wertschöpfungsketten pflanzlicher und tierischer Produkte. Die vorliegende Arbeit untersucht schwerpunktmäßig den möglichen Einsatz plasma-induzierter Oberflächen- und Inhaltsstoff-interaktionen zur selektiven und gezielten Modifikation von Sekundärmetabolitprofilen in Erbsenpflanzen und technofunktionellen Eigenschaften von Erbsenmehlen und –proteinen. Weiterhin wurden die über die plasma-gestützte Modifikation pflanzlichen Materials gewonnenen Erkenntnisse übertragen auf tierische Rohstoffe aus essbaren Insekten und zusätzlich die mikrobielle Dekontamination, als ein weiterer Schwerpunkt bei der Insekten-verarbeitung, mit einbezogen. Die Arbeiten zur Erzeugung gewünschter Modifikationen umfassen die detaillierte Charakterisierung ausgewählter Rohstoffe, die Auswahl geeigneter KADP-Anlagen und korrespondierender produktspezifischer Prozessparameter, sowie die prozessbegleitende Erfassung notwendiger Plasmaeigenschaften. Die hierzu notwendigen detaillierten Untersuchungen der plasma-induzierten Effekte wurden nach einem Top-down-Ansatz unter der Nutzung geeigneter analytischer Methoden, die Einblicke in mögliche zugrundeliegende Mechanismen liefern, von der makroskopischen zum molekularen Ebene durchgeführt. Das makroskopische Untersuchungslevel beinhaltete Methoden zur Erfassung der Qualität (Farbe/Textur), Zusammensetzung (Protein-/Fett-/Trockensubstanzgehalt) und Mikrobiologie (Oberflächen-/Gesamtkeimzahl), begleitet von Untersuchungen technofunktioneller (Wasser-/Fettbinde-/ Emulgiereigenschaften) und Proteineigenschaften (Löslichkeit, Wasser-/Fettbinde-/Emulgiereigenschaften) als auch der Proteinstruktur (Öberflächenhydrophobizität/Fluoreszenzeigenschaften/CD-Spektroskopie) auf der mikroskopischen und struktureller Ebene, bis hin zur Proteinzusammensetzung (SDS-PAGE/Tryptophangehalt/Aminosäurezu-sammensetzung) auf der molekularen Ebene. Die Ergebnisse liefern eine wissenschaftliche Grundlage zur gezielten Nutzung der KADP-Technologie zur Funktionalisierung und Modifizierung proteinreicher Lebensmittelkomponenten und könnten folglich zur bioökonomischen und ressourceneffizienten Produktion hochwertiger trockener Proteinprodukte beitragen, da Proteinfunktionalität eine wesentliche Rolle in Produktverbesserung und -entwicklung und der Nutzung alternativer Proteinquellen spielt. Aus der Identifizierung produktspezifischer Eigenschaften und der Beurteilung der Behandlungseffektivität hinsichtlich des gewünschten Prozessziels wurden wichtige Erkenntnisse zum langfristigen Ziel der Kombination von KADP mit Prozessschritten etablierter Verarbeitungsketten gewonnen. Erste Versuche zur Behandlung komplexer Lebensmittelmatrices unter Nutzung einer Plasmaanlage, die sich zum Einsatz im industriellen Maßstab eignen würde, zielten auf die Entwicklung innovativer kosteneffizienter Kombinationen aus Plasma- und Trocknungsverfahren ab. Das Potential des KADP-Verfahrens in Zukunft zu einem Routineprozess der Lebensmittelindustrie zu werden zeigt sich in allen Teilen der Arbeit, die folglich einen wesentlichen Beitrag zur erfolgreichen Zulassung der KADP-Technologie im Lebensmittelsektor beiträgt.

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List of abbreviations

a_w-value	Water activity
BSA	Bovine serum albumin
β-LG	Beta-lacto globulin
CAPP	Cold atmospheric pressure plasma
CD	Circular dichroism
CFU	Colony forming units
cPF	Commercial pea flour
DBD	Dielectric barrier discharge
DC	Direct coupled
DM	Dry matter
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization of the United Nations
FTIR	Fourier transform infrared spectroscopy
GC-MS	Gas chromatography-mass spectrometry
HPP	High pressure processing
INP	Leibniz-Institut für Plasmaforschung und Technologie e.V. Greifswald
KADP	Kaltes Atmosphärendruckplasma
LCD	Liquid crystal display
MLD	*Musculus longissimus dorsi*
NaCl	Natrium chloride
OMF	Oscillating magnetic fields
PAW	Plasma activated water
PBS	Phosphate buffer solution
PEF	Pulsed electric fields
PF	Pea flour
POD	Peroxidase
PPA	Plasma processed air
PPF	Pea protein flour
PPI	Pea protein isolate
PPO	Polyphenol oxidase
PPW	Plasma processed water
RF	Radio frequency

RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDBD	Surface dielectric barrier discharge
SEM	Scanning electron microscopy
T-DF	Defatted *Tenebrio* flour
TEAP	Triethylammonium dihydrogen phosphate
T-F	*Tenebrio* flour
TFI_{max}	Maximum tryptophan fluorescence intensity
T-HPF	High protein *Tenebrio* flour
TVC	Total viable count
UV	Ultra violet
VUV	Vacuum ultra violet
WPI	Whey protein isolate
XRD	X-ray diffraction
λ_{max}	Maximum emission wavelength

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1. Introduction

Modern day society is characterized by an increasing health consciousness and the interest in the role of food for maintaining and improving human well-being and consumer health has grown over the past decades. Besides their nutritional and sensory properties, thus, foods are currently seen as active and protective agents. Therefore, and in consideration of a continuously growing population, the food industry must continually adapt to meet both the demands in terms of nutrition and consumer expectations. Especially with regards to food production and processing, innovation is required in order to fulfill the emerging challenges of global food supply meeting the demands of nutritional intake and the complexities of the modern food chain. As food safety remains a major challenge in the food production chain, further new food safety intervention strategies are required to manage food safety across the global food supply chain. Also with regard to reducing losses and wastes [1] in the worldwide food production efficient strategies, which maintain the product quality characteristics while extending shelf-life and reducing the microbiological safety risks of food products, are required.

Fresh-cut horticultural products stand out, inter alia, as convenient and novel minimally processed foods that cover most needs of a modern lifestyle, as they combine technical content with an innovative food concept [2]. At the same time, nonthermal processing technologies for food preservation evolved in search of alternatives to conventional thermal processing, as these technologies have the potential to address the demands of the consumers in delivering high-quality processed foods with an extended shelf-life, which are additive-free and have not been subjected to extensive heat treatment. Consumers are often satisfied by the fresh-like characteristics, minimized degradation of nutrients, and the perception of high quality due to the relatively mild conditions of most nonthermal processes when compared with heat pasteurization. Consumers demand for foods, which are minimally processed, meet their nutritional and taste desires and further require minimal preparation. Besides being concerned about the ingredients within the consumed foods, they are more and more conscious about processes being exploited along the food chain.

Thermal processes indeed effectively inactivate microorganisms and enzymes, but their negative impact on color, flavor and nutritional quality of foods has not always met consumer demands. In this context, nonthermal technologies have been designed commonly defined as preservation treatments, which are effective at ambient or sub-lethal temperatures, thereby minimizing negative thermal effects on nutritional and quality parameters of foods [3]. Most of the topics related to nonthermal processing technologies, which up to date received most attention (high pressure processing (HPP), irradiation, ultrasound, ozonation and electrical methods such as pulsed electric fields (PEF), light pulses, electrolyzed oxidizing water and oscillating magnetic fields (OMF)), have been well-researched and a wealth of information is available [4-7]. Nowadays, the field of nonthermal food technologies can be extended by cold atmospheric pressure plasma (CAPP) as an innovative food technology.

Based on phase transitions occurring by continuously supplying energy to a system, various

states of matter are recognized. Besides the 'traditionally' known solid state, liquid and gas phase and the more recently found low-temperature state (Bose-Einstein condensate, [8]), and high-temperature states, such as plasmas, exist. The majority of matter in the visible universe, as stars, interplanetary and interstellar medium, is in the plasma state. Lightning, sparks and St. Elmo's fire are examples for natural terrestrial plasmas. Northern Lights, or aurora, are caused by the energy in radioactive rays from the sun (solar wind) colliding with electrons in oxygen and nitrogen in the atmosphere. Free electrons resulting thereof are then deflected by the earth's magnetic field to create a plasma effect [9].

Applications of plasma processing are found in science, technology, and industry, while this technology has been used first in the 1970s for etching semiconductor materials [10], then its application within the evolving computer industry, particularly for the fabrication of miniaturized circuits, went on during the 1980s. Currently, the plasma technology is widely used in the field of semiconductor and data-storage manufacturing, in particular for deposition and etching process applications. Similar process applications are found in the manufacture of flat-screen LCD televisions and photovoltaic solar panels. Figure 1 gives an overview of the current applications of gas plasma technologies in various areas of science, technology and industry covering many aspects of everyday life and almost all major industries. Plasma technologies offer a wide spectrum of possible treatments of materials and thus, are used in the polymer and textile industries for surface modification. Further, the plasma technology has gained increasing importance in nanotechnology, especially for the synthesis of nano-particles, and also represents an alternative technology for gas phase de-pollution of volatile organic compounds emitted by various industries. Liquid-phase destruction of pollutants in industrial effluents is further being tested. Besides all the technical and industrial uses, in the fields of analytical chemistry, plasmas generally represent useful tools for optical spectroscopy and mass-spectrometry [11]. Hereby the application of plasma in the area of biology and medicine has been identified as one of the most exciting and multidisciplinary fields, as plasma offers an effective approach for medical treatment of various skin injuries and diseases, *e.g.*, wounds, bacterial superinfections, and fungoid infections. Recently, gene transfer using discharge plasma has attracted attention. The emergence of plasma biomedicine caused a shift in plasma application from treatment of inanimate to living or cellular objects.
In food processing, the direct application of so-called "cold plasma", as well as semi-direct or indirect treatment with thermal plasma is of interest, as these can be used to treat the food at temperatures below 70 °C [12]. Due to its nonthermal character and its operation under atmospheric pressure, CAPP could offer a suitable approach for the treatment of heat-sensitive foods, as fruits and vegetables. In this respect, the CAPP technology offers high microbial inactivation efficiency at low temperatures (often below 50 °C) allowing it to extend shelf-life, and thereby substantially contributing to improving the efficiency of supply chain. The active plasma-immanent species act rapidly and access the entire food surface in most cases, as they are characterized by high diffusivity. As the application of the CAPP technology is free of water or solvent and further most cold plasma sources require only a low energy input, it is also considered environment friendly and energy efficient.

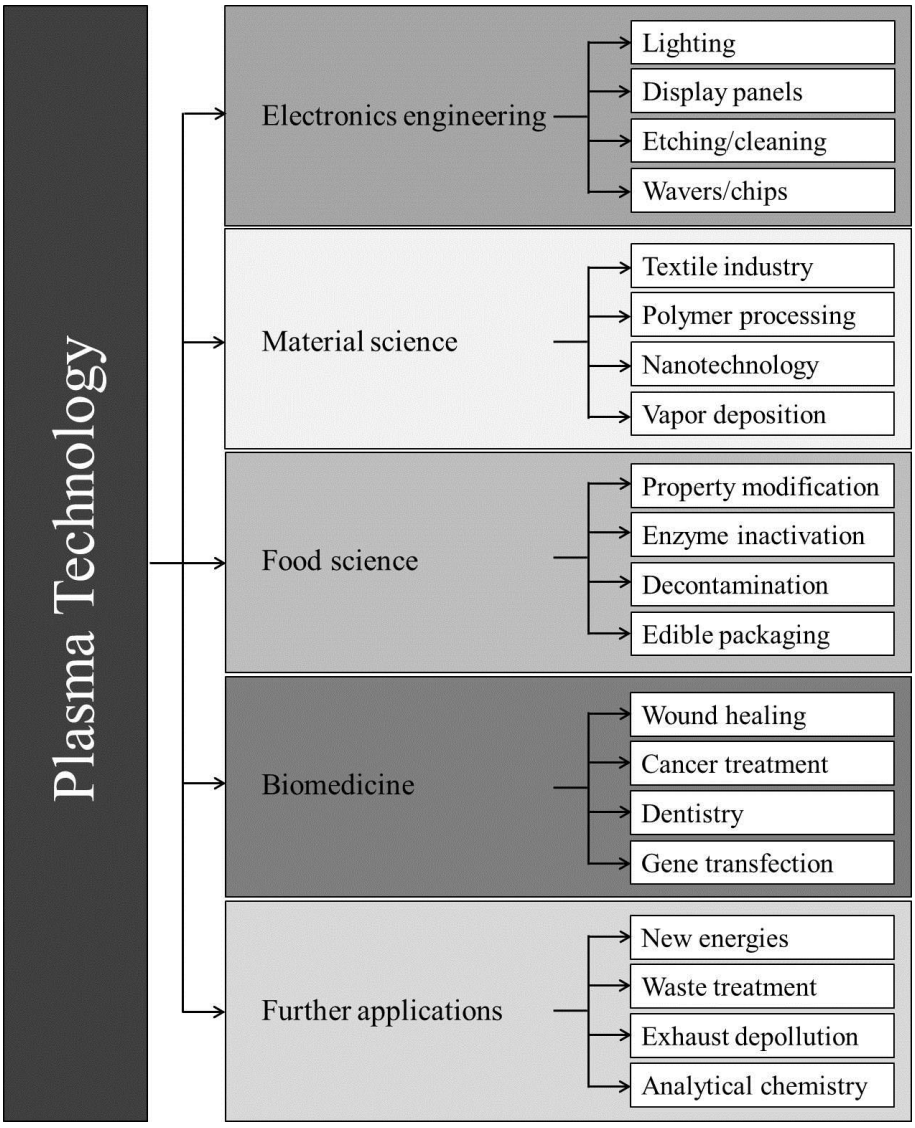

Figure 1: Overview of nonthermal plasma applications in various areas of science, technology and industry.

Due to the relatively early state of specific technology development of CAPP in the food sector, its impacts on sensory and nutritional qualities of treated foods are largely unexplored. Optimization and scale up to commercial treatment levels require a more complete understanding of the plasma-induced chemical processes and the antimicrobial modes of action, which vary for different CAPP systems. As this area of CAPP technology shows promise, it is the subject of active and intense research.

1.1 Cold atmospheric pressure plasma: Definitions, generation and sources

Ice melts and changes to the state of liquid water; water evaporates and turns into water vapor. This pattern of transition between the three states of solid, liquid and gas is familiar, but there is another fourth material state (Fig 2). Although the generation of a plasma from the gas phase is strictly spoken not a real phase transition, plasma is, due to its unique physical properties distinct from solids, liquids and gases, often referred as 4^{th} state of matter. In

physics and chemistry, plasma is defined as a completely or partly ionized gas containing free electrons, positive and negative ions, neutral atoms, and neutral or charged molecules, and is further characterized by its temperature, different types of radiation (*e.g.* UV) in various wavelength regions, and by electric fields. Due to the presence of charged carriers, plasmas are conductive and strongly respond to electromagnetic fields [13].

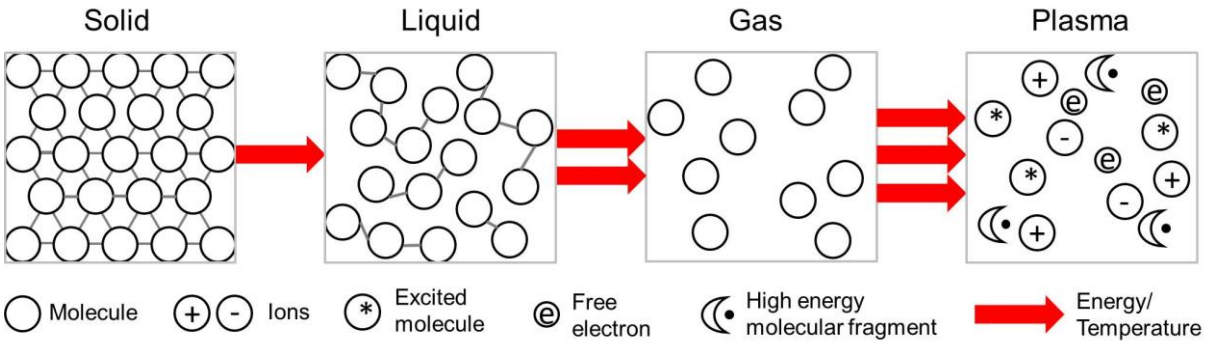

Figure 2: The four fundamental states of matter.

The first, who named ionized gas "plasma", were Lewi Tonks and Irving Langmuir [14] defining a state of matter, in which a significant and equal number of atoms and/or molecules are electrically charged or ionized. Despite all of the constituent particles being charged, typically, the plasma itself has no overall charge. In contrast to ideal gases, ionized gases exhibit a dynamic, collective behavior, which is caused by long-range interactions. These interactions originate from electromagnetic coupling between the charged particles (Coulomb attraction and repulsion), and electric and magnetic collective perturbations (due to free charge carrier motions). As the presence of charged ions means that plasma is highly electrically conductive and responds strongly to magnetic and electric fields, biasing the collective behavior by applying suitable electromagnetic fields leads to a temporary spatial confinement of the plasma, and thereby allows a certain controlling of the plasma dynamics. The existence of multiple temperature regimes, related to different plasma particles and degrees of freedom, represents another fundamental plasma characteristic. Plasmas are commonly classified in terms of their thermodynamic properties, by which thermal plasmas and nonthermal plasmas, also regarded as plasmas in thermodynamic equilibrium and non-equilibrium plasmas, can be discriminated (Fig. 3, [15]). Thermal plasmas are characterized by (nearly) total ionization of the system, in which the collision frequency is high with respect to the particles transit time on the plasma scale length. Consequently, the efficient energy transfer in electron-ion collisions leads to thermalization of the different particle species to the thermodynamic equilibrium temperature with the energy content equally shared among vibration, rotation and translation energies (equipartition theorem). All species present are in the local thermal equilibrium, *i.e.* all species have the same mean free kinetic energy (temperature). Fragmentation reactions to atomic levels of all organic molecules present in the plasma are induced by the extremely high energy content. Such plasmas are produced in fusion experiments and plasma arcs with temperatures above 10^4 K [16]. Due to this, the application of these so-called hot plasmas is often limited.

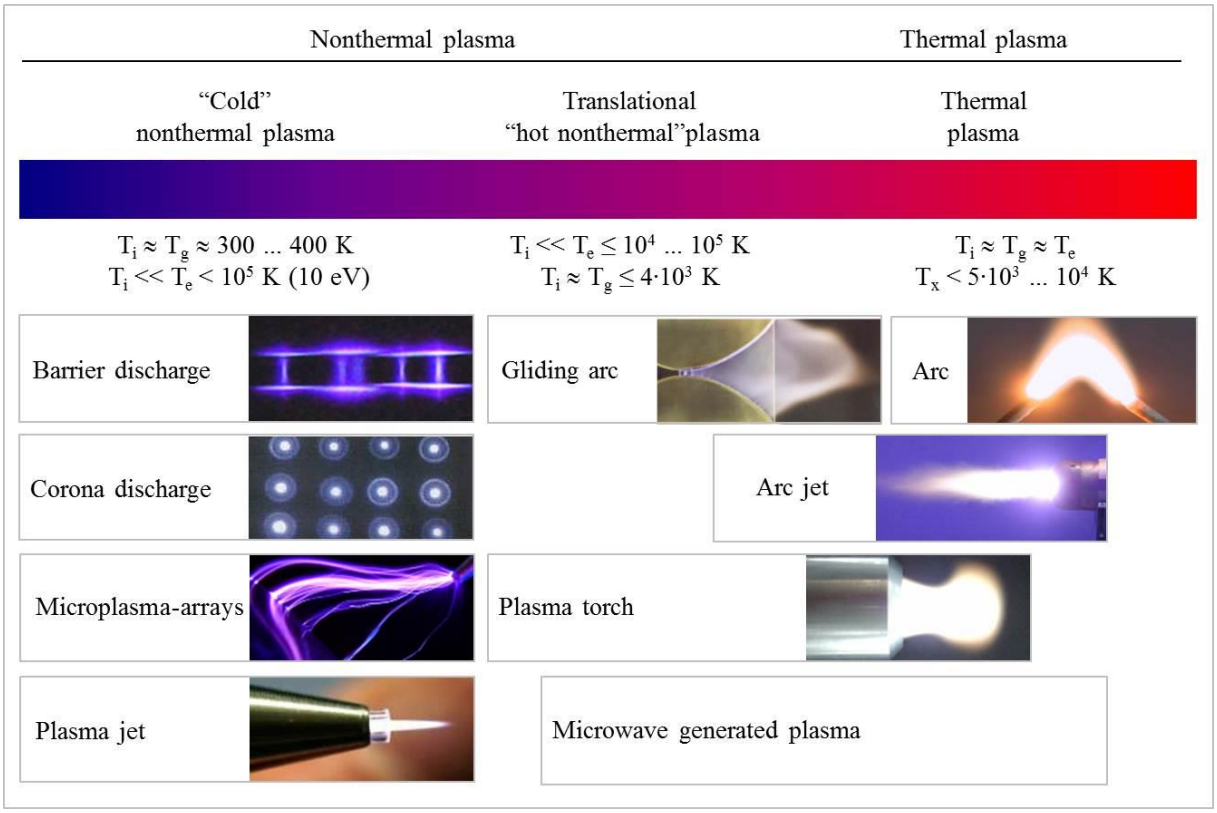

Figure 3: Division of different types of plasma by the temperature of plasma species, adapted from Weltmann & von Woedtke [17].

In contrast, partially ionized plasma is in a thermodynamic non-equilibrium state. While heavy weight particles (neutrals and ions), representing the main plasma compounds, may be at almost ambient temperature, electrons are found to have temperatures in the order of 10^4 K. Most of the coupled energy is primarily released to the free electrons, which exceed the temperatures of the heavy plasma components by orders of magnitudes. The plasma temperature is determined by interactions of neutrals or ions with walls, as well as by collisions of the electrons with the background gas and to the walls, and is, due to the low electron heat capacity and density, generally close to room temperature. The interactions among different species (*e.g.* electron-ion or electron-neutral collisions) may be increased either by increase of the pressure or the density of the electrons. Consequently, the electron and gas temperatures tend to equilibrate and converge to similar values. Even in non-equilibrium plasmas the gas temperature can increase to some 10^3 K. Such plasmas are called "hot nonthermal plasmas". Besides thermal and nonthermal plasmas, translational plasmas represent an optional third group. In actual fact, translational plasmas are nonthermal plasmas, but their field of application for translational plasmas is almost identical to the application area of thermal plasmas, since their gas temperature is much higher than the temperature of typical nonthermal plasmas [18].

Generating plasma artificially, it can be ignited at low or atmospheric pressure by adding energy to a gas, *e.g.* air, argon or helium. In principal, this can be done regardless of the nature of the energy source employed. Therefore, plasma generation can be of mechanical, radiant, chemical and thermal origin or occur under the influence of electric and electromagnetic fields with sufficient high field strength. Thermal plasmas can be generated,

e.g. by inductive coupling of high-frequency fields in the MHz range (inductively coupled plasma), by microwave coupling in the GHz range (plasma torch, *e.g.* PLexc©) or by direct coupling (conductive, coupling, arc discharge). For technological and technical applications, the most common method of plasma generation is applying an electric field to a neutral gas. With exceeding a certain threshold (breakdown field strength) by the electric field applied, a gas discharge and, thus, plasma is formed. As electrical energy has been shown to be most suitable for balancing energy losses, electrical discharges are the most commonly used discharges for generating nonthermal plasmas. Alternatively, plasma can be generated by the interaction of an electron beam with gaseous medium. The gas temperature and type of plasma can be controlled via the electrical current flowing through the plasma or by the frequency of the voltage applied and is further affected by the frequency of the electric field. Transition between different types of discharges occurs upon increasing currents. Electrical current remains low in "cold" nonthermal plasmas, as dielectric barrier discharges (DBD) and corona discharges, whereas at certain threshold values the transition to an arc discharge is achieved with increasing current values and, thus, increasing temperatures of gas and electrodes. Usually, the electrical breakdown process is completed in the time interval of 10^{-8} to 10^{-6} s. At low alternating fields, the position of anode and cathode exchange after each half cycle, whereas at higher frequencies covering the radiofrequency and microwave range, there is a change in plasma mechanism, which diminishes the role of electrodes and is used in so-called plasma torches. With increasing frequencies, the temperature of the gas also increases. Consequently, such high-frequency plasmas usually belong to translational or thermal plasmas [12].

The various geometries of the reactors and the number and location of the electrodes employed (electrode systems involving two or multiple electrode configurations or electrodeless systems), make the number of plasma reaction chambers almost countless and technological applications of plasmas formed in these sources are numerous. Just as diverse as the discharge devices are, together with plasma and applicator parameters, a wide range of adjustable parameters is provided (Table 1). The efficiency of the method is further strongly dependent on product-specific properties. Consequently, the specific energy input, heating of the product, and temperature distribution are as important as material properties, composition, geometry, and whether the material being treated is uniformly shaped, in pieces, powdered, or a liquid. Surface characteristics as pores, capillary openings, and the availability of water are influencing the plasma-induced effects and efficacy of the treatment. Besides the process temperature, as a particularly suitable parameter for comparative assessment of plasma methods, parameters as electron energy distribution, plasma composition, and the specific energy input may also be used [19].

Further, plasmas can be operated in a continuous or in a pulsed mode, in closed (cavities) or open structures (*e.g.* surfatron, plasma jet). Depending on the electric and electromagnetic field used for plasma generation, a whole branch of plasma sources, as inductively and capacitively coupled installations, can be recognized [20]. The generation of plasma at atmospheric pressure with temperatures of about 30 to 40 °C represents the basis for treating living cells, tissues, and other heat sensitive material. A new field, "Plasma Medicine",

combining plasma physics with life science and medicine developed rapidly [15].

Table 1: Technical characteristics and influencing parameters to describe plasma treatment, adapted from Schlüter et al. [12].

Individual systems	Category	Example parameters
System	Plasma parameters	Type of plasma generation Geometry Voltage Current Pressure Gas mixture
	Applicator parameters	Chamber volume Treatment pressure
	Product parameters	Treatment area/volume Dosage Process temperature
Plasma	Radiation	Spectral power distribution
	Charged particles	Electron density Ion energy distribution Ion density
	Neutral particles	Type Density Lifetime Reactivity Temperature

New plasma sources and devices were introduced for different applications enabling the generation of plasmas at atmospheric pressure and close to ambient temperature, thus, allowing nonthermal treatment conditions [16]. Meeting these essential requirements, nonthermal atmospheric pressure plasma offers the potential for its reactive components to be used to inactivate microbial contaminants at low temperatures, primarily on food surfaces [21]. The antimicrobial effect of CAPP was demonstrated in numerous studies including human pathogens and even bacterial endospores on food surfaces, as well as in liquid food matrices (see section 1.2.1). The first laboratory-scale test series on the use of plasmas in the food sector were mainly studying possibilities of inactivating undesirable microorganisms on heat-sensitive foods, as conventional thermal decontamination methods are more or less unsuitable for products, such as fresh fruit and vegetables, meat, and eggs. Plasma treatment is also regarded as a potential alternative to other chemical (*e.g.* chlorine treatment) or physical methods (*e.g.* high-pressure, pulsed electric fields, ionizing irradiation). Plasma processing is advantageous due to its

i. high efficiency at low temperatures, its
ii. precise generation suitable for the intended use, the
iii. just in time production of the acting agent, and the

iv. very low impact on the internal product matrix.

Further, the application of CAPP is free of water or solvent addition. Several research works have identified the capability of cold plasma technology in decontaminating fresh produce and, offering this potential, recently CAPP has been added to the list of nonthermal processes.

In food processing, the direct application of nonthermal, as well as semi-direct or indirect treatment with thermal plasma is of interest, as these can be used to treat the food at temperatures below 70 °C (Table 2). For CAPP applications in the food sector, preference should be given to processes carried out at atmospheric pressure (plasma jet, dielectric barrier discharges), as the atmospheric conditions allow continuous process control and do, compared to applications at reduced pressure ($p < 1013$ mbar) or low pressure ($p < 10$ mbar), not accelerate undesirable phase transitions.

Table 2: Overview of different types of cold plasma, adapted from Schlüter et al. [12].

Type	Description	Examples
Direct	Plasma is in direct contact with the substrate Interaction based on irradiation (VUV, UV), charged molecules, radicals, and reactive species	Plasma jet DBD
Semi-direct	Distance between plasma and substrate much larger than the mean free particle path No interaction with charged particles Antimicrobial effect based on irradiation, long-lived radicals, metastable and inhibitory substances	SDBD with gap Sterrad process with plasma-activated hydrogen peroxide
Indirect	Irradiation with VUV, UV No reaction with plasma particles Plasma is used to treat gas or liquids	UV lamps Ozone generator Plasma-processed air (PPA) Plasma-processed water (PPW)

The composition of the plasma and its contained immanent species strongly depends on the individual plasma source and the underlying principles of plasma generation, the process parameters employed and the process gas used. This technical diversity results in large variation in the antimicrobial efficiency and related product-process interactions of the respective plasma [20]. There may be several other applications in relation to food systems, which still remain unexplored. With respect to this, treatment conditions suitable for the application of CAPP on a vast range of food produce can be found for the different plasma sources [22] and need to be optimized with regard to the defined process goals.

Due to the very small dimension of available plasma sources, to this end majority of studies reported the use of plasma jets for treatment of foods. Baier *et al.* [22] investigated the use of a plasma jet operated with argon gas for treatment of corn salad leaves, while Bermúdez-Aguirre & Barbosa-Cánovas [23] utilized a plasma jet array operating with argon for decontamination of lettuce, carrots and tomatoes. Atmospheric pressure plasma jets area nonthermal, spatially uniform glow plasma discharges operating at atmospheric pressure. Typically, plasma jet systems consist of a ceramic nozzle with an inner coaxial needle

electrode mounted in the center of the nozzle, and an outer grounded ring electrode placed at the nozzle outlet (Fig. 4). A radio-frequency (RF) or direct coupling (DC) generator is connected to the center electrode via a matching unit in order to match the impedance of the generator to that of the discharge for maximizing the power transfer and minimizing the reflected RF power [19]. The discharge operates on a feedstock gas (noble gases, *e.g.* argon with the addition of molecule gases, *e.g.* oxygen or nitrogen), which flows between the two electrodes. By applying an electric field, a bulk plasma starting from the tip of the needle electrode and expanding outside the nozzle into the ambient air is generated between the electrodes. Highly energetic electrons are present at the entire length of the jet or so-called plasma afterglow [24]. Working at atmospheric pressure and without a closed treatment unit the process gas effluent is gradually mixed with the surrounding air before impinging on the substrate. In dependency on the gas flow rate, the working gas composition, and the power applied, the plasma afterglow has a length of 10 mm. The cross section of the afterglow is limited by the inner diameter of the nozzle outlet to about 7 mm, which enables the point-wise and simultaneous treatment of small spots. The application area further depends on the distance between the sample surface and the plasma nozzle, as well as on the gas composition. During CAPP application to liquids, the sample is continuously mixed by the gas flow, in this case, every volume element comes into contact with the plasma applied.

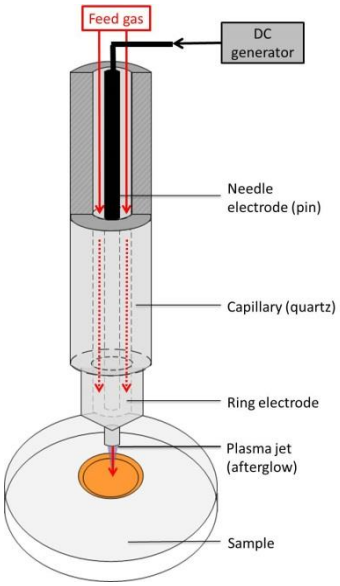

Figure 4: Schematic depiction of a plasma jet (kINPen 09, INP Greifswald) operating at atmospheric pressure conditions.

As previously mentioned, often noble gases are employed for generating plasmas, which increases the cost of treatments. In order to adopt the CAPP technology for food industry, the operating cost of the process gas further play an important role. An ideal gas for such treatments would be ambient air. In view of the throughput in product processing at a scale of tons per hour, this represents a major problem for the assessment of the CAPP technology regarding its application in food production. The choice of plasma source should not distract from realistic appropriateness for up-scaling. First attempts in this direction can be found in the field of plasma medicine, where indirect plasma treatment within a remote exposure

chamber was applied to disinfect larger workpieces, such as medical instruments [25].

An alternative plasma source for treatment of foods allowing the treatment over large volumes is the use of dielectric barrier discharge (DBD) set-ups. DBD plasmas enable the treatment over large volumes in air and discharge gaps, when sufficiently high potential difference is maintained across the gas gap. The DBD is an alternating current discharge in nonthermal equilibrium typically generated between two electrodes, whereas at least one dielectric limits the discharge current. Depending on the process gas used and operating voltage applied, the distance of the electrodes alters from microns to centimeters. Commonly used electric operation parameters range from line frequency to several MHz; some kV ignition voltage, and power consumption of some W/dm^2 electrode area are used. Depending on the setup, a volume or a surface discharge is generated discerning two discharge modes: diffuse and filament discharge.

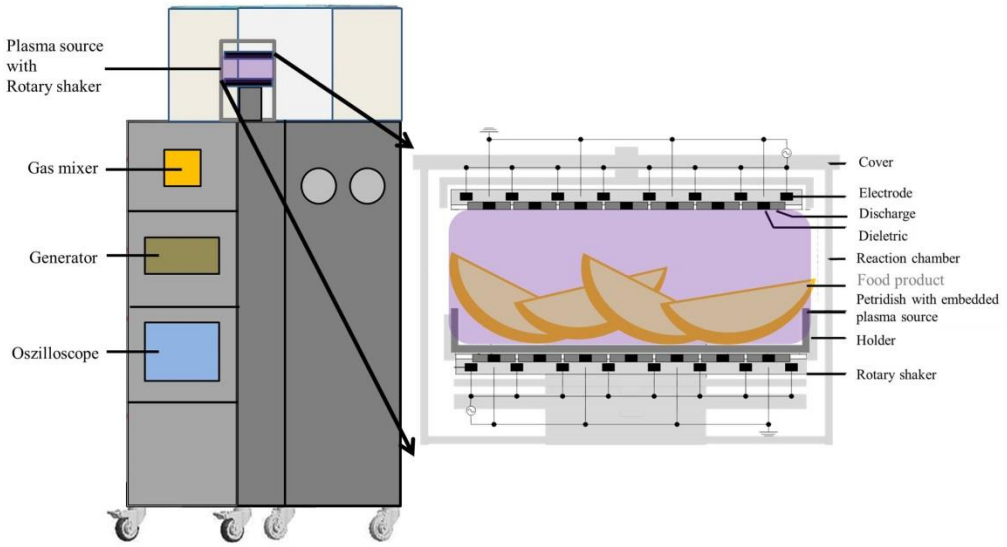

Figure 5: Schematic depiction of a DBD plasma device operating at atmospheric pressure conditions (adapted from 'LegUMAX', INP Greifswald).

The process gases used and the electrical operation of the discharge represent crucial parameters for the operation mode, whereas nearly every combination of gases ranging from noble gases over air or water vapor, up to special admixtures of precursors can be used. Further, surface DBD (SDBD) plasma setups are applicable for the treatment of foods, especially in liquid or solid dry powder or granular form. Oehmigen *et al.* [26] used a SDBD setup specially designed to fit into 60 mm diameter petri dish in order to analyze the impact of an indirect discharge on microorganisms in solution and demonstrated that depending on the exposure time the pH value of the solution dropped to 2.78 for 0.85 % NaCl solution, whereas the pH value phosphate buffered saline solution (PBS) remained at 7 even after 30 min plasma exposure. The authors attributed the result to the formation of NO_x species inside the discharge and interpreted the acidification of non-buffered solution as a consequence of the formation of nitrous acid (HNO_2) and nitric acid (HNO_3). Further, DBD setups are advantageous in treatment of produce inside sealed packages eliminating the risk of post-process contamination. The use of in-package plasma technology for treatment of foods is

well established [27, 28]. Hereby, the package material itself serves as the dielectric material and helps to limit the charge transported, thereby permitting the generation of a stable discharge and further eliminating the need for additional charge barriers.

As formerly mentioned, the choice of plasma source should not distract from realistic appropriateness for up-scaling. Another attempt in this direction can be found in using indirect plasma treatment within a remote exposure chamber. Contrary to small plasma apparatuses, indirect treatment chambers provide the advantage to eliminate all difficulties such as cracks, crevices or cavities on food surfaces. A new approach to enable plasma treatment of larger goods is the use of more energetic plasma sources, as microwave-driven plasma torches. By using microwaves for plasma ignition, high plasma densities of up to 10^{13} cm^{-3} and high gas temperatures in the range of 10^3 K are induced at a typical frequency of 2.45 GHz and a wavelength of 12.24 cm. When cooling down the plasma gas from high temperatures transferred at the site of ionization to non-thermal conditions, its exhausts can be fed into a treatment chamber and applied to heat-sensitive foods. Investigating the use of generated "plasma processed air" (PPA) and "plasma processed water" (PPW) for decontaminating larger foods was objective of a number of works. The use of microwave-driven plasma torches is a well-established technique to generate plasma and has, due to its unique advantages, attracted the interest of a range of scientists in recent years [29-31].

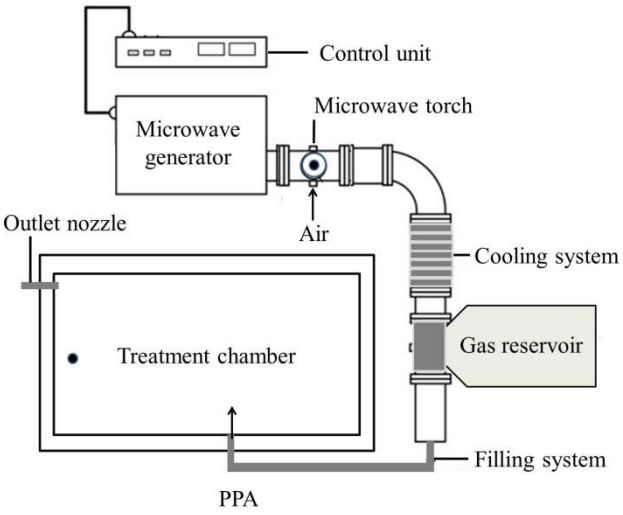

Figure 6: Schematic depiction of a microwave-driven plasma device for the generation of PPA.

Baier *et al.* [32] tested the PLexc-system (PLexc®: Plasma excited, INP Greifswald) for its suitability to treat corn salad plants and for its feasibility on voluminous types of produce investigating the critical parameters for a practice-oriented application of thermal air plasma for effective, sustainable and gentle sanitation on whole pieces of different kinds of fresh produce. Cooled down to nonthermal treatment conditions, PPA was indirectly fed to fruit and vegetables within a remote exposure treatment chamber. The device consists of a microwave generator, a plasma source, a compressor, a humidifier, a device for oxidizing NO, a process chamber, a vacuum pump, and of a control and regulating unit. A microwave generator supplies the plasma source with microwave energy generating hot plasma from the supplied air under atmospheric pressure (burst mode with an ignition/pause-cycle of 20x5s/7s). The

plasma gas emerging from the plasma source is cooled within a specified time to the point that a plasma-activated gas mixture with an NO_2 content of at least 0.5 % is formed by means of the device for oxidizing NO. This plasma-activated gas mixture is humidified with water in a humidifier and further admitted into a process chamber containing the item to be treated [33]. Then, the process chamber can be filled with the PPA at ambient temperature (about 22 °C) resulting in nonthermal conditions within the treatment chamber. Consequently, the process of ionization of the process gas takes place spatially separated from the actual process of application by the fact that the plasma-reactive species are generated both in the ionization phase and in the ensuing recombination and cooling phase. A direct plasma treatment does not take place, as only the plasma-activated gas mixture formed therefrom is used for treatment. This method for indirect CAPP application therefore is characterized by

i. generating a plasma with air as process gas, which forms reactive nitrogen and oxygen species,
ii. oxidation of NO to NO_2 at temperatures below 400 °C forming a plasma-activated gas mixture with an NO_2 content of at least 0.3%,
iii. bringing this plasma-activated gas mixture into contact with water (PPW), and
iv. bringing the generated gas mixture into contact with the products to be treated (PPA).

Generating PPA at a rate of up to approximately 4 m^3/h with air as the working gas, and with small amounts of water, as well as with only one plasma source having a microwave power of approximately 2 kW, this method also operates extremely economical and efficient. Since the plasma process and the sterilization process are separated from one another, several process chambers may be filled in succession with PPA using one plasma source. In this way, it is possible to treat larger goods either individually in a high-speed process or in large quantities in a batch process using one or more chambers [34].

1.2 Product-plasma interactions in food processing

The product-process interactions during CAPP are manifold and strongly dependent on product properties, and the plasma application system. Specific knowledge about the appropriate interactions is necessary to control the impact of CAPP on the matrix treated and must be taken into consideration for tailor-made process designs.

Regarding the plasma treatment of food materials, it should be kept in mind, that neither the plasma process nor the product processed can be considered as static systems. On the contrary, it is to be assumed that product and process interact with each other fairly strong (Fig. 7). Penetration depth and product temperature during the application of plasma for instance are strongly dependent on processing parameters, *e.g.* power input, gas composition, and distance between the plasma source and the product on the one hand, but are also heavily affected by for instance the water availability (a_w-value) and the surface structure of the food matrix being treated. Further, plasma-induced reactions, as the entry and/or recombination of reactive species into the food matrix, and reactions with product ingredients, as well as a decrease in pH are product specific and have to be taken into consideration mainly for liquid

and water rich systems. Whereas CAPP treatment of solid foods, where penetration of reactive species is limited, mostly influences the food surface, and especially the plasma treatment of dry materials primarily induces modification, functionalization, and change in the structure of the surface, liquid food matrices represent a quite reactive environment. Based on their interaction with water and other molecules, reactive oxygen species (ROS) and reactive nitrogen species (RNS) initiate multiple chain reactions in liquids, resulting in a great variety of different species.

However, the fact that merely the presence or absence of water strongly influences the plasma treatment, clearly shows the complexity of plasma-process interactions, which need to be taken into consideration when developing plasma processes based on reliable scientific knowledge.

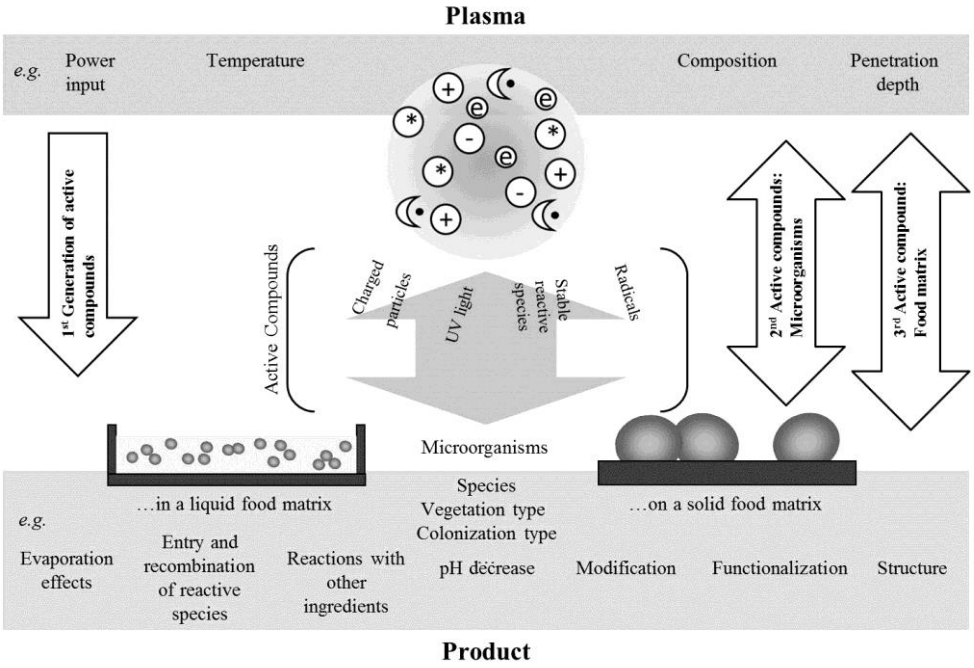

Figure 7: Product-process interactions that may take place during plasma treatment of liquid and solid food matrices.

In general, there are two major differences between applying plasma on solid and liquid media: the penetration depths or contact surface between plasma and food, and the chemistry/physics initiated by ROS and RNS. When applying CAPP on solid foods, the treatment is, due to the very low penetration depth of the plasma-immanent species, usually limited to their surface. The ability of plasma species to penetrate into solid foods depends on several factors, as the food composition, its water content, and its porosity. It was shown in a handful of studies, which dealt with the penetration depth of that reactive species including ROS, RNS, ozone, and UV that plasma can only penetrate some µm deep into biofilms. Studies conducted by Xiong *et al.* [35], who investigated the penetration depth of a plasma jet into biofilms formed by *Porphyromonas gingivalis* bacteria using a confocal laser scanning microscope, evidenced that the plasma was capable of inactivating the bacterial cells up to a depth of 15 µm. In a similar study, Pei *et al.* [36] inactivated *Enterococcus faecalis* in a

25.5 µm biofilm using a handheld air plasma jet.

In case of direct treatment using plasma jet systems, the composition of the plasma, its flow rate, the distance between the nozzle of the jet and the matrix treated, as well as the food surface represent additional limiting factors regarding the penetration depth. Hydrogen peroxide for instance has a half-life of 1 ms and is comparably stable, whereas other ROS, such as singlet oxygen and hydroxyl radicals, have half-lives of 1 µs and 1 ns, respectively, leading to very limited penetration depths (Table 3). However, in most cases, a limited penetration depth is advantageous, if the defined process goal of applying plasma to the surface is to achieve a gentle microbial decontamination. If so, a low penetration depth helps to retain the majority of nutrients inside the food, while achieving proper surface decontamination, particularly for foods with a high surface-to-volume ratio.

Table 3: Properties of selected reactive oxygen species, adapted from Surowski et al. [37].

Reactive oxygen species	Half-life	Present in	Penetration depth (diffusion coefficient 10^{-9} m^2/s)
Hydroxyl radical ($OH^{\cdot}$)	1 ns	Plasma, air, and liquid	1 nm
Singlet oxygen ($^1O_2$)	1 µs	Plasma, air, and liquid	30 nm
Superoxide (O_2^{-})	1 µs	Plasma, air, and liquid	30 nm
Hydrogen peroxide (H_2O_2)	1 ms	Air-liquid interface, and liquid	1 µm

A contrary behavior is found in liquid foods. Consequently, penetration depth is not as limited as during treatment of solid systems, as in this case every volume element comes into contact with the plasma applied (or at least with subsequent reaction products). Thus, if plasma is applied to liquid foods, possibly all other contained components are harmed. Therefore, the focus of process optimization has also to be put on retention of other food constituents at the same time and thus, represents a key challenge.

Oehmingen *et al.* [26] investigated the generation of chemical species during atmospheric-pressure plasma treatment of aqueous liquids and subsequent diffusion and convection processes in the liquid volume. They visualized acidification, as well as generation of nitrite in the water treated by a surface dielectric barrier discharge under atmospheric conditions in ambient air using color forming reactions, and evidently demonstrated that

i. the changes of liquid composition by atmospheric pressure plasma treatment are initiated by reactions at the plasma/gas-liquid interface, and

ii. the depth effectiveness of plasma treatment is realizable, if long-lasting chemical species are generated, and diffusible liquid phases are available.

The subsequently ROS-initiated chemistry has several consequences, as for instance water dissociation reactions with electrons, once plasma comes into contact with water molecules. The major ROS formed in liquids are hydroxyl radicals ($OH^{\cdot}$), which can be generated by electron dissociation and electron attachment, as well as by thermal dissociation, ion and metastable pathways. OH radicals are able to subsequently react and form hydrogen peroxide (H_2O_2). In dependency of the pH, H_2O_2 can be very stable and might remain active in the

liquid for a much longer time than the plasma exposure itself. Further, hydroperoxy radicals (OOH·**)** or superoxide ($O_2^{-\cdot}$) in the presence of OH radicals, which can also be comparably long-lasting, may be formed. The occurrence of these ROS and its formation in liquids has been reported in literature [38, 39]. The term "plasma-activated water (PAW)" is widely used in this context referring to the plasma-treated liquid, which retains its antimicrobial properties for a long time [40-43].

These reactions do not play a role in dry and solid foods; they are rather influenced by different plasma-induced fundamental processes, such as etching, deposition, recombination, de-excitation, and secondary emission from solids. Especially atomic oxygen is capable of triggering etching effects leading to the formation of volatile compounds, as CO_2 and H_2O. Etching of surfaces in general is accompanied by weight losses deriving from the ablation of the first (atomic) layer of the surface and also with surface chemical reactions [44].

1.2.1 Microbial inactivation

The application of CAPP enables the effective inactivation of a wide range of microorganisms including spores [45, 46] and viruses [47]. In medical and bioengineering, the plasma technology is used for sterilization and decontamination of heat-sensitive object surfaces. Research on lowering the microbial count using atmospheric plasmas have mainly been carried out on carrier materials like metal, glass, paper, and plastics, such as polypropylene and polyethylene terephthalate, providing results that show the high potential to inhibit or inactivate microbes.

The use of antimicrobial and sterilizing properties of plasma was first introduced towards the end of 1960s, when Menashi patented a corona-based "plasma sterilization" in 1968 [48] and Laroussi developed a large-volume dielectric barrier discharge plasma inactivation system in 1995 [49]. Since then, considerable research has been performed on the plasma-underlying mechanism of microbial inactivation. Plasma agents contribute to the lethal action, as various reactive species of plasma interact with the biological material and cells to cause permanent changes finally leading to inactivation. During plasma treatment, microorganisms are exposed to an intense bombardment by the radicals in plasma most likely provoking surface lesions. That the living cell cannot repair the lesions and induced pores sufficiently faster may partially explain the observations wherein cells are in many cases destroyed very quickly. Pelletier [50] termed this process "etching". Using humid air plasma additionally provokes a marked acidification of the medium in addition to the induction of pores and surface lesions. The combination of highly energetic plasma species with a nonthermal treatment mode makes nonthermal plasmas particularly suitable for decontamination in food processing including the dry disinfection of packaging materials, food surfaces (*e.g.* meat, poultry, fish and freshly harvested horticultural produce), granular and particulate foods (*e.g.* dried milk, herbs and spices), and sprouted seeds.

The effects of plasma can be quite selective, meaning tuneable between damage to pathogenic organisms without damage to the host, or activation of different pathways in different

organisms [51]. By varying the process parameters involved in plasma generation, a multitude of mechanisms can be actuated, which may act individually or synergistically. Plasma-immanent species, as hydroxyl radicals, hydrogen peroxide, ozone, singlet oxygen, superperoxide, nitrogen oxide, as well as UV radiation, act on the microorganisms and affect various macromolecules, as DNA, proteins, and lipopolysaccharides. UV-induced DNA damage, photodesorption, and radical etching have been described as mechanisms underlying the inactivation of microbes [52]. In the case of low pressure plasmas, the former is regarded as the main factor for successful sterilization [53-55], whereas etching was identified as the key inactivation mode for plasma treatment at atmospheric pressure [56, 57]. (Lethal) damage of microorganisms is attributed to the oxidation of cell components, accumulation of charged particles on the surface of the cells, lowering of the pH value with loss of pH regulation, breakdown of the membrane potential and energy generation [45, 58, 59]. The main radicals formed in plasma, when using air as the process gas, are OH• and NO•, which can undergo ensuing reactions in aqueous media, thus significantly lowering the pH value [29, 60, 61]. Microbial inactivation, when using atmospheric plasma within packaged foods, is attributed to ozone and NO_x species formed in the plasma [62-64]. Besides by the variation of plasma process parameters, the inactivation efficiency is also influenced by the bacterial density, vegetation and colonialization type, and the bacterial species on the surface being treated, as well as by the physiological state of the bacterial cells. Further, the type of food being treated must be taken into consideration as one of the factors that influence the microbial inactivation effectiveness of CAPP [65]. Regarding the treatment of bulky and irregularly shaped food, restricted volume and size of the food should be considered in selecting the proper plasma setup. Taking into account these aspects, plasma processing parameters can be tailor made in order to meet the product specific requirements.

1.2.2 Surface modification

The plasma technology is used in areas where joining of materials or precise modification of their surfaces is important in order to bond materials together or to change their surface properties to suit the required needs in various industrial branches. CAPP is able to modify virtually any surface and therefore, offers numerous applications [66], as

i. precision cleaning of small and micro components,
ii. activation of plastic components prior to gluing, painting, etc.,
iii. etching and removal of different materials, and
iv. coating of components with barrier layers, hydrophobic and hydrophilic layers, friction reducing coatings, etc..

New applications are constantly evolving and thus, the plasma technology has established itself in all areas of industry, as it exhibits decisive advantages compared to other methods, such as flame treatment or wet-chemical treatment. Many surface properties can only be obtained using this universally applicable method, which is online-production capable and can be fully automated. Plasma further offers an environmentally friendly process being almost independent of product geometry, as powders, small parts, plate materials, non-wovens,

textiles, tubes, hollow bodies, circuit boards, etc. can be treated, which are not mechanically altered and further subjected to minimal thermal loads. In many industrial applications the plasma technology combines very low running costs with high process and work safety and further represents a particularly rational process [67].

During application of CAPP to solids, many fundamental processes take place at the plasma-substrate interface (Table 4), whereas the effect of plasmas on a given material is determined by the chemistry of the reactions between the surface and the reactive species present in the plasma [68]. At low energies, as typically used for plasma-surface treatment, the induced plasma-surface interactions only modify the surface of the solid material. Further, the effects are confined to a region only several molecular layers deep and do not change the bulk properties of the substrate. The resulting surface modification or functionalization depends on the surface composition of the product exposed to CAPP treatment, as well as on the process gas used. Gases, or mixtures of gases, used for plasma-surface treatment of polymers, can include air, nitrogen, argon, oxygen, nitrous oxide, helium, tetrafluoromethane, water vapor, carbon dioxide, methane, or ammonia, whereas each gas produces a unique plasma composition and results in the induction of different surface properties.

Table 4: Plasma-surface interactions, adapted from Braithwaite [68].

Plasma-surface interactions	Reactions
Etching	$AB + C\ (solid) \rightarrow A + BC\ (gas)$
Deposition	$AB\ (gas) + C\ (solid) \rightarrow A\ (gas) + BC\ (solid)$
Recombination	$e^- + A^* \rightarrow A$
De-extraction	$A^* \rightarrow A$
Secondary emission	$A^* \rightarrow A + e^-\ (from\ surface)$

In general, the CAPP treated surface is subjected to bombardment by fast electrons, ions, and free radicals, which is accompanied by continued electromagnetic radiation emission in the UV-Vis range enhancing chemical-physical reactions in order to obtain the desired functional and aspect geometries. The most prominent plasma-induced surface effect is the secondary electron emission from solids related to consequently increasing surface roughness [69].

The surface energy can be increased very quickly and effectively by plasma-induced oxidation, nitration, hydrolyzation, or amination. In dependency of the plasma working gas and the chemistry of the treated surface, substitution of molecular moieties into polymer surfaces can make them either wettable or totally non-wettable. Depending on the chemistry and process variables, mainly three competing surface processes proceed simultaneously: ablation, crosslinking, and activation. Ablation is similar to an evaporation process, where the bombardment of the polymer surface by energetic particles (*i.e.*, free radicals, electrons, and ions) and radiation breaks the covalent bonds of the polymer backbone resulting in lower-molecular-weight polymer chains. With shortening of long molecular components volatile oligomer and monomer byproducts ablate and are swept away either with the vacuum-pump exhaust or with the gas flow when using a plasma jet system. Because of the high etching rate, argon and oxygen plasmas are particularly useful in ablating polymers [70]. Further, CAPP can be used for the surface functionalization of polymers (Fig. 8), polymer degradation, and

cross-linking [71]. Surface functionalization refers to the formation of functional groups on the polymer surface (*e.g.*, oxygen- and nitrogen-containing groups) occurring, when hydrogen atoms on polymer chains form carbon radicals and subsequently causing oxidation or nitration. Crosslinking means bond breaking occurring on the polymer surface. Without free-radical scavengers, bond formation with nearby free radicals on a different chain (crosslink) can be induced.

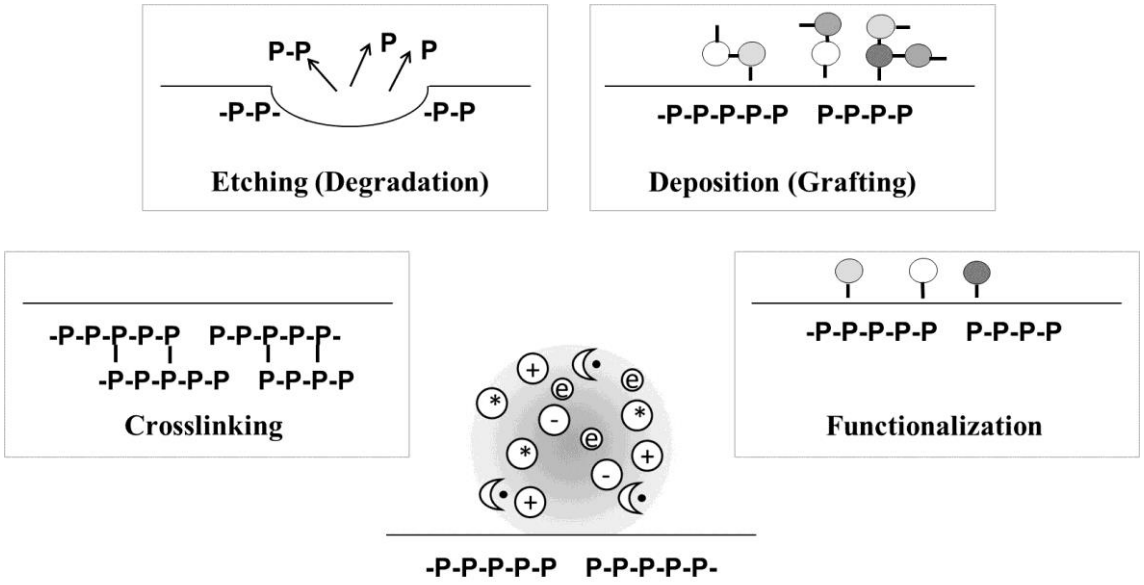

Figure 8: Surface modifying processes that may take place during plasma treatment; adapted from Bazaka et al. [72].

The process of activation is characterized by the plasma-induced replacement of different atoms or chemical groups at the polymer surface. Similarly as described for ablation, exposure of the surface to energetic species abstracts hydrogen or breaks the backbone of the polymer under the creation of free radicals. In addition, dependent on the process parameters, plasma contains very high-energy UV radiation, which creates additional similar free radicals on the polymer surface. As they are thermodynamically unstable, these free radicals quickly react with the polymer backbone, itself or with other free-radical species present at the surface to form stable covalently bonded atoms or more complex groups.

Owing to the surface modifying and functionalizing effects of plasma, studies in the area of life sciences and related fields have so far concentrated on the possibility of controlled ablation of harmful substances, *e.g.* the removal of bacterial endotoxins from the surface of medical instruments. Possible adverse effects of plasma treatment on foods have rarely been investigated, although it is well-known that CAPP also modifies the surface structure of food related materials at the micro- to nanometer range [71, 73] and hereby allows to chemically and physically modify surface characteristics of polymeric materials without affecting their bulk properties. With regard to the modification of surface properties of polymeric materials, CAPP treatment of food materials is advantageous for several reasons, as its use is free of hazardous solvents; there is uniformity of treatment, and there is no generation of thermal damage when in contact with materials [74, 75]. Therefore, plasma-specific effects on polymer surfaces may offer an innovative approach for the modification of biopolymers in the

food sector.

Despite the fact that cold plasma applications are working at moderate temperatures, regardless of the nature of the substrates or plasma gases, each plasma enhanced surface functionalization is accompanied by etching processes. The number of reports on plasma treatment of food surfaces is limited and a whole branch of different plasma sources were used in the studies. For instance, plasma processing of plant material for food may change the structure of the food matrix, which is of great importance for the bioavailability of phytochemicals ingested in the matrix [76, 77]. In order to elucidate whether ion bombardment and subsequent oxidation reactions cause measurable changes on the surface of plants following exposure to CAPP treatment, Grzegorzewski *et al.* [78] analyzed the surface wettability of plasma-treated lamb's lettuce by means of contact angle measurement. The surface wettability of leaves was increased. Whereas the surface of the pristine leaves was hydrophobic (contact angle of 88), exposure to direct plasma treatment gradually reduced the contact angle until a value of 34 was reached (180 s exposure) and the surfaces became more hydrophilic. The authors did not unambiguously attribute the large reduction in contact angle upon plasma exposure to a plasma-induced formation of oxygen-containing functional groups on the sample surface [75, 79], but also to the degradation of the cuticle layer, which is composed of cutin and covered by miscellaneous hydrophobic cuticular and epicuticular waxes [80]. This could be particularly critical, as the plant's surface barrier usually hinders most microorganisms to penetrate and spoil the inner tissues. It is, however, as well conceivable that ablation of the waxy layer offers distinctive advantages for further preservation steps. Attachment to the hydrophobic plant surface is usually believed to limit contact between chlorinated water and microbial contaminants [81]. Changing the plants surface properties can, thus, impede microbial attachment and spoilage, or improve conventional sterilization procedures [82].

Oh *et al.* [83] reported the effects of CAPP on the physical properties of edible biopolymer films prepared from agricultural process byproducts using defatted soybean meal in order to (i) evaluate the effects of CAPP using various plasma process gases on the physical properties of the film, (ii) determine the optimal plasma conditions for improving the tensile and moisture barrier properties of the film, and (iii) investigate the effects of packaging smoked salmon with plasma-treated film on salmon quality factors including color, lipid oxidation, hardness, and biodegradability, while stored at 4 °C. Results derived from this study suggest the use of CAPP for improving the applicability of edible films in food packaging. The authors conclude that the CAPP technology has the potential to improve the properties of edible films or other agricultural process byproduct-based films and thus, enhances their applicability as food coating and wrapping materials.

Misra *et al.* [84] demonstrated the potential of plasma in enhancing the surface hydrophobicity of freshly baked biscuits evident from the increased spread area of vegetable oil. As compared to currently used method, for a given volume of oil, up to 50 % more spreading of oil could be achieved within a few seconds of the process. For this particular application it could be advantageous that the induced effects fade over time. Nevertheless, detailed investigations regarding the effects of cold plasma on the chemical constituents of the

biscuits and on the very probable oxidation of fat is urgently required.

1.2.3 Impact on food components

It is a general problem that currently little is known about the effect of plasma treatment on food model substances. Depending on other contained ingredients, a whole branch of plasma-initiated reactions can finally result in the oxidation of constituents such as proteins, carbohydrates, and lipids. The following chapter gives an overview about the most important food constituents and their reactions with ROS, including proteins, lipids, and carbohydrates.

Proteins

Proteins carry out a great variety of biological functions and consist of a high number of amino acids connected via peptide bonds forming a polypeptide chain, which represents the primary structure of every protein. The protein secondary structure describes the spatial arrangement of these amino acids depending on the hydrogen bonds located between the peptide bonds forming different types of structure: α-helices and β-sheets (Fig. 9). The tertiary structure of a protein further describes the spatial arrangement of the polypeptide chain, which is determined by forces and bonds existing between the amino acid side chains, such as disulphide and hydrogen bonds, as well as hydrophobic, ionic, and van der Waals forces. The aggregation of different proteins is stabilized via *e.g.* hydrogen or ionic bonds, but also by covalent bonds and is called quaternary structure.

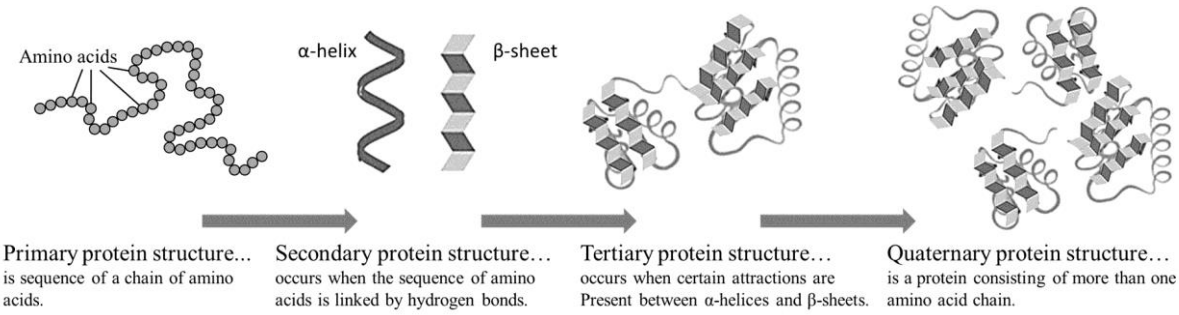

Figure 9: Primary, secondary, tertiary and quaternary structure of proteins.

The biological function of a protein is determined by its spatial structure and thus, any structural modification of proteins leads to a modification or inhibition of their functionality. A range of plasma-immanent species is capable of undergoing structure-modifying reactions with proteins, as the oxidation of amino acids by ROS. Sulfur-containing and aromatic amino acids are preferred for ROS attacks.

Cysteine and methionine, as two representatives of the former group, are susceptible to reactions with a wide range of ROS, particularly $OH^{\cdot}$ and $^{1}O^{2}$. The formation of disulphides, such as cysteine by oxidation of the thiol group (–SH) contained in cysteine (Fig. 10), as well as the formation of mixed disulphides can be triggered by ROS.

Another example is the reversible oxidation of methionine (Fig. 11), which is supposed to act as an endogenous antioxidant protecting the active site or other sensitive domains in the

protein from getting oxidized to methionine sulfoxide [85].

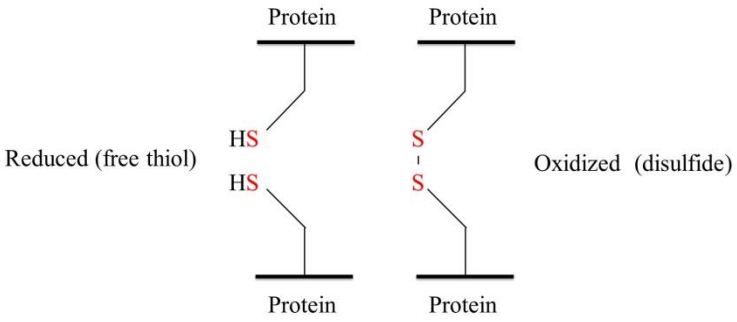

Figure 10: Disulfide (sulfur-sulfur) linkages between two cysteine residues.

Tryptophan, as one of the aromatic amino acids, can be oxidized by ROS under formation of N-formylkynurenine and has also been suggested to act as an antioxidant to a certain extent [85]. Oxidative attacks can also results in carbonylation (Fig. 12), an irreversible formation of free carbonyl groups (C=O) in amino acids, such as arginine, histidine, lysine, proline, threonine, and tryptophan. Nguyen & Donaldson [86] showed that carbonylation can be the reason for the inactivation of enzymes, such as catalase.

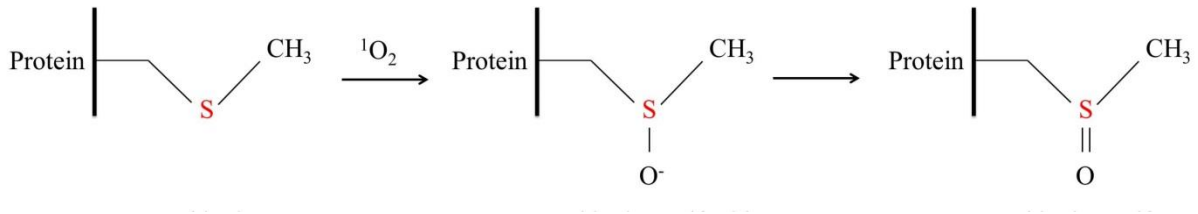

Figure 11: Reversible oxidation of the sulfur of methionine to methionine sulfone.

Most of the authors investigating the plasma-indicated effects on proteins used enzymes as model systems, as all of the reactions described between ROS and amino acids are also applicable on enzymes, which are macromolecular biological catalysts and also belong to the group of proteins in most cases. Further investigations regarding the plasma-mediated enzyme degradation included trying to find explanations for occurring changes in activity and to identify the reactive species involved. Fluorescence spectroscopy and circular dichroism (CD) spectroscopy are typical methods being used in order to identify changes of the enzymes' structure.

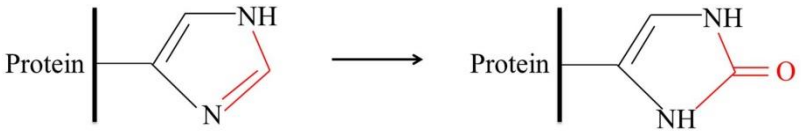

Figure 12: Carbonylation, an irreversible formation of free carbonyl groups, in amino acids.

The former is widely used for the quantification of aromatic amino acids, such as tyrosine and tryptophan. Further, the intensity, as well as location of their characteristic peaks, has been shown to be a helpful indicator for identifying and studying occurring enzyme modifications.

CD spectroscopy is an excellent tool for determining changes of the different structure fractions (α-helices, β-sheets, turns, and random coils).

Lipids

In general, lipids are esters of moderate to long-chain fatty acids, which -depending on the number of double bonds existing between their carbon atoms- can be saturated, monounsaturated, or polyunsaturated. Plasma-immanent species, as ROS (particularly $OH^{\cdot}$, $^1O_2$, and $HOO^{\cdot}$), which combine with a hydrogen atom to make water and a fatty acid radical, are the most notable initiators for lipid oxidation in living cells. This also refers to lipid oxidation in foods. The primary targets for ROS are C–H-bonds (methyl groups) preferably located between double bonds, as the energy input needed in order to abstract a hydrogen atom is much lower there than compared to CH-bonds bound elsewhere [87]. Consequently, the more double bonds a fatty acid contains, the more susceptible it is against homolytic ROS attacks. For this reason, linoleic acid (18:2) and α-linolenic acid (18:3) containing two and three double bounds, respectively, are fatty acids sensitive to ROS initiated oxidation. The overall mechanism of lipid oxidation consists of three phases: (i) initiation, the formation of free radicals; (ii) propagation, the free-radical chain reactions; and (iii) termination, the formation of nonradical products [88] and is accompanied with the formation of peroxy radicals, which in turn also break CH-bonds under formation of hydroperoxide and another radical. This chain reaction can be stopped either by the recombination of radicals or by the effect of antioxidants, which act as radical scavengers.

Lipid peroxidation has a huge impact on sensory attributes of food, as it is accompanied with the generation of off-flavors. Volatile carbonyl compounds, such as pentanal, cis-4-heptanal and trans-6-nonenal formed depending on the fatty acid oxidized, are responsible for rancid, fishy, fatty, or metallic flavors.

CAPP-induced changes to milk and free fatty acids contained therein were investigated by Korachi et al. [89]. An effect on polyunsaturated fatty acids was detected, whereas the total free fatty acid concentration did not change significantly. The authors conclude that the occurring changes may be attributed to dehydrogenation caused by oxygen radicals and further found that the levels of long-chain fatty acids decreased, while the levels of short-chain fatty acids increased. The generation of shorter chain fatty acids as a result of the impact of reactive species has also been reported in other studies and could be attributed to hydrolytic effects. ROS are capable of initiating lipid peroxidation and produce hydroperoxide, which may be subsequently converted into secondary oxidation products, such as aldehydes and shorter chain fatty acyl compounds [90, 91]. The exposure of polyunsaturated fatty acids to ROS ($HOO^{\cdot}$, $O_2^{\cdot-}$, and $^1O_2$) was found to result in the generation of shorter fatty acids [92, 93].

Carbohydrates

Sugar alcohols, such as mannitol and sorbitol, have shown to scavenge $OH^{\cdot}$, as carbohydrates are particularly susceptible to the action of $OH^{\cdot}$ and thus, protect more vital cellular components from being oxidized [94, 95]. It was reported that sucrose and different polyols were the most effective scavengers and that their OH scavenging activities increase with

increasing sugar alcohol concentration. Isbell & Frush [96, 97] found that aqueous alkaline hydrogen peroxide solutions degrade aldohexoses almost quantitatively to 6 mol of formic acid, and aldopentoses to 5 mol, whereas the mechanism behind formic acid formation is explained as stepwise degradation of aldoses: the addition of a hydroperoxide anion to the aldehyde modification of the sugar and subsequent decomposition of the adduct to formic acid and the next lower aldose. In addition, the authors found that the reactivity of the different hexoses and pentoses can be directly linked to their tendency to exist in the aldehyde form. As a consequence, glucose, which has the lowest proportion of the aldehyde form, showed the lowest reaction rate of the six aldohexoses studied. Ribose has the highest reaction rate in the pentose series, because it has the highest proportion of the aldehyde form.

Food matrices are commonly composed of different amounts of macronutrients as proteins, lipids, carbohydrates, and water representing, together with micronutrients, such as minerals and vitamins, essential components for the human diet. When observing the plasma-mediated reactions of these components isolated from others, and when the composition of reactive species applied is known, their behavior during plasma exposure might be comparably easy to predict. However, real food matrices are very complex multicomponent targets with different percentages of constituents which influence the impact and induced process-interactions of the plasma applied.

2. Motivation and objectives

It is known that the treatment of food with CAPP offers many more possible application options beyond the gentle sanitation of heat-sensitive food surfaces. In order to establish innovative CAPP applications in the food sector and to integrate CAPP treatments for the tailored increase in value of agricultural produce into existing value-added chains, the detailed knowledge in specific plasma-induced effects is crucial.

The research work documented in the present thesis aimed at identifying and analyzing plasma-induced product-process-interactions with the overall goal to reveal potential applications of the CAPP technology along food value-added chains. Starting from the literature-known CAPP-induced effects on surfaces and secondary metabolites of agricultural plant produce, this work was mainly motivated by the following issues and aspects:

i. It is known that nonthermal plasmas can modify and even destroy a wide spectrum of surfaces and organic compounds, whereas there are many uncertainties with regard to the interaction of plasma-immanent reactive species with plant surfaces and phytochemical compounds. Therefore, on the one hand, it is of particular interest to elucidate and understand the basic plasma-product interactions in order to avoid nutritional degradation or any other undesired effects in future CAPP applications. On the other hand, the tailored controlling of desired plasma-induced effects in turn may be exploited for tailored applications in related scientific disciplines. For this purpose, the first part (Section 3) of this work deals with tailor-made applications of CAPP derived from detailed investigations of individual plasma-induced effects, reactions and interactions examined by using model systems. This includes the application of CAPP for evidencing a radial strain gradient in cuticles from apple fruit utilizing the surface modifying plasma effects to develop a plasma-assisted preparative method, as a literature search found that evidence for such a strain gradient was lacking (Section 3.1). Further, the capability of using air plasma to induce the formation of nitro-phenolics in aqueous solutions was investigated, as the nitration of aromatic compounds is one of the most fundamental reactions in organic synthesis, which provides key organic intermediates or energetic materials and, therefore, represents an immensely important process in pharmaceutical and chemical industry (Section 3.2).

ii. Based on the outcomes of plasma-induced effects on surfaces and reactions with plant secondary metabolites evidenced by using model systems, two related scientific issues arose: Firstly, is it possible to use the plasma-induced effects on plant secondary metabolites in a targeted manner in order to selectively modify ingredient profiles in plant tissues by using the CAPP technology as a kind of stress-inducing treatment? Secondly, is it possible to use the plasma-induced surface modifications as a tool for the targeted modification of proteins' tertiary and secondary structure of proteins and, thus, their functionality in dry raw materials, intermediates and products from alternative plant protein sources? For this purpose, the second part (Section 4.1) of this work aimed at investigating the integration of the CAPP technology into the value-

added chain of legumes considering both of the former mentioned application possibilities as selected examples (Sections 4.1.1 and 4.1.3). As the detailed knowledge on the raw material including composition, protein- and techno-functional properties and in which ways these specific material properties are affected during production and further processing are indispensable prerequisites for the tailored improvement of selected properties, the studies on the application of CAPP, as an innovative alternative to conventional treatment, include the detailed characterization of the raw material used (Section 4.1.2).

iii. The plasma-assisted modification of proteins in dry bulk materials may further be transferable to other raw materials, as edible insects, representing an animal-based alternative protein source (Section 4.2). Up to now, the use of edible insects in the food sector is poorly investigated, thus triggering the need for the detailed characterization of the raw material, as well as first examinations regarding the general processing and fractionation of insect flours (Section 4.2.1). Due to the current state of research, the comparison of effects induced by innovative CAPP processing was compared to conventional thermal treatment using dry heat (Section 4.2.2). As insect larvae are often spoiled with microorganisms on their surface and in their intestine, besides flour and protein modification, the microbial decontamination is one key issue, which needs to be considered in the value-added chain of edible insects for the production of food. The third part of this work focuses on the cross-value chain transfer of knowledge, wherefore the CAPP treatment of flour seems to be most promising to meet both aforementioned process goals.

iv. Plasma-protein interactions are also transferable to enzymes, which are macromolecular biological catalysts and also belong to the group of proteins in most cases. As naturally occurring compounds in food, most of them are undesired with respect to food processing, since they catalyze reactions, which negatively affect food quality characteristics. Especially polyphenol oxidase (PPO) and peroxidase (POD) are well known for being involved in enzymatic browning reactions and resultant losses in nutritional value. Therefore, inactivation of these enzymes is beneficial and thus, part of numerous CAPP-related studies. Much research has been conducted regarding the application of CAPP to inhibit undesired enzymatic reactions related to food processing, but there is a lack in investigations regarding the applicability of CAPP for the treatment of real food systems. Therefore, the fourth and final part (Section 4.3) of this work aimed at providing insights into the CAPP treatment of complex food matrices by using a plasma device, which is promising for the application in industrial scale, and further represents an initial approach towards process integration and combination by investigating the effectiveness of CAPP treatment as a pre-drying procedure for the inactivation of PPO and POD in fruits and vegetables (Section 4.3.1).

3. Individual ingredient and surface reactions: Tailor-made applications

As plasma treatment creates the prerequisites for innovative new manufacturing processes, material combinations and products, it has been engaged in intensive research into future application areas continuously developing new solutions and new industrial applications. However, the unique characteristics of plasmas and their induced chemical and surface reactions should result in much broader applications.

Since knowledge on plasma applications from other scientific and industrial fields can be transferred to agricultural and food science, detailed new findings gained in agricultural and food science may also offer insights into mechanisms for the development of targeted applications in the field of other related sciences, technologies and industries. Plasma-induced interactions with surfaces and ingredients offer a broad range of applications in analytical and chemical areas. Reactions and effects caused by CAPP treatment, which are probably unwanted in agricultural or food engineering, may be used in a targeted manner in other areas. However, real food matrices are very complex multicomponent targets with different percentages of constituents, which influence the impact and induced process-interactions of the plasma applied. A totally different behavior of the target compound may be induced solely by the addition of just one component. Carrying out broader analysis of plasma-induced effects in food related (single-component) matrices is a basic prerequisite for

i. gaining deeper knowledge regarding specific plasma-induced processes taking place during plasma-interactions with specific surfaces and components in order to
ii. optimize relevant process parameters for either
iii. the prevention of undesired or
iv. the tailored controlling of desired plasma-induced effects,

which in turn may be exploited for a specific purpose. One example of such controversial effects and reactions is the plasma-induced oxidation of lipids. Whereas it is usually unwanted during processing of food, some studies were already dealing with using the CAPP application as a tool to accelerate lipid oxidation in order to simulate slow alteration processes [98, 99]. Besides detailed knowledge regarding the plasma-induced mechanism of lipid oxidation gained hereby, both studies conclude that, in comparison to commonly applied thermally based tests, plasma exposure is capable of accelerating lipid oxidation in a realistic manner and, in turn, propose CAPP as an analytical tool for simulating accelerated lipid oxidation. There are other complex effects related to plasma-specific surface and ingredient interactions being observed, when treating agricultural produce, which need to be further investigated and which further may offer the potential for the targeted application in analytical and preparative approaches.

For instance, the plasma-induced removal of plant material layers from surfaces may be used to analyze material layers and related specific characteristics. As formerly mentioned, the

number of reports on plasma treatment of food surfaces is limited. Grzegorzewski, et al. [78] observed the time-dependent changes of lamb's lettuce (Valerianella locusta) leaf surface morphology caused by plasma-induced erosion phenomena. In order to elucidate whether ion bombardment and subsequent oxidation reactions cause etching of the upper epidermal layer and cell ablation, the authors analyzed the plants surface morphology by means of SEM. They found that the surface of plasma-treated leaves becomes rough and granular structures disappear with increasing exposure time, while untreated lettuce leaves were characterized by wide areas with thick platelets and small-sized granular structures. Further, the degradation of the upper cuticle layer, which is composed of cutin and covered by miscellaneous hydrophobic cuticular and epicuticular waxes [80], was found to be as well feasible and was evidenced. These effects could be used for the targeted ablation of epicuticular wax, cutin, as well as upper cell layers from plant surfaces. Consequently, these plasma-induced surface modifications will change the structure of the food matrix during plasma processing of plant material. The targeted ablation of cell layers and waxes from plant surfaces could offer an innovative preparative tool necessary in the field of surface analytical and morphological scientific issues. One possible design of experimental set-up, which was used in parts of this work to ablate and subsequently analyze wax and cell layers from fruit cuticle surfaces in a targeted manner, is presented in figure 13.

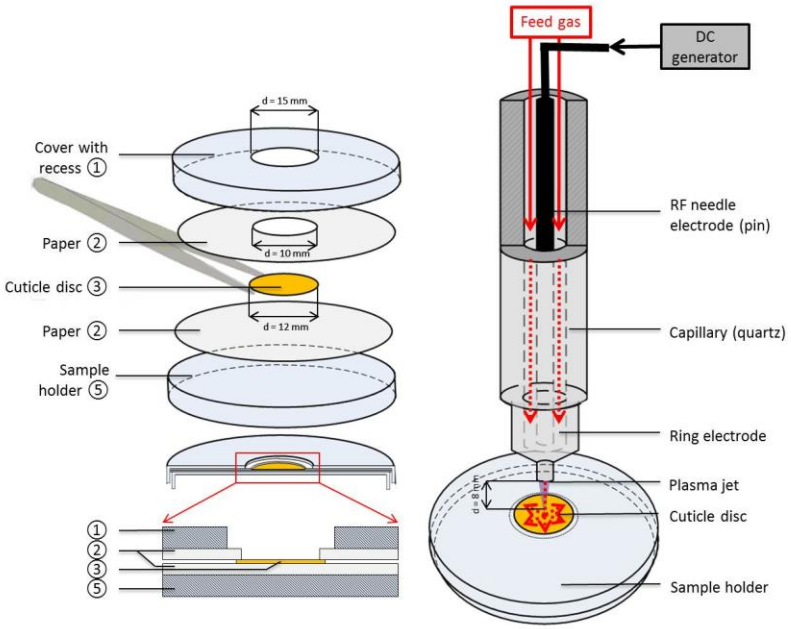

Figure 13: Design of experimental plasma-jet set-up applicable to ablate wax and cell layers from fruit cuticle surfaces in a targeted manner.

A contrary behavior is found in liquid foods. Consequently, penetration depth is not as limited as in case of treating solid systems as in this case every volume element comes into contact with the plasma applied (or at least with subsequent reaction products). Thus, if plasma is applied to liquid single component model food systems plasmas may not only be able to increase the efficiency of traditional chemical processes but could further offer alternative approaches to otherwise inaccessible reaction pathways using conventional chemical synthesis.

The same study conducted by Grzegorzewski, et al. [78] further aimed at investigating the interactions of plasma-immanent reactive species with secondary plant metabolites. As formerly mentioned, lamb's lettuce was exposed to an atmospheric pressure plasma jet for this purpose. Various chemically reactive plasma compounds, all contributing synergistically, make the underpinning plasma chemistry rather complex. As CAPP, with its huge variety of reactive species and resulting interactions, is already complex itself, the complexity of reactions occurring becomes even more complex when the gas discharge comes into contact with multicomponent systems as complex food matrices. As it is a general problem that currently little is known about the effect of plasma treatment on food model substances, changes in the phenolic profile of lambs' lettuce leaves were compared to effects determined in experiments with pure solid substances in order to compare these to the influence on the food matrix. The experiment set-up allowed analyzing the plasma-induced modification of the model compounds limited to the surface of the model matrix with a subsequent limit in penetration depth of the plasma.

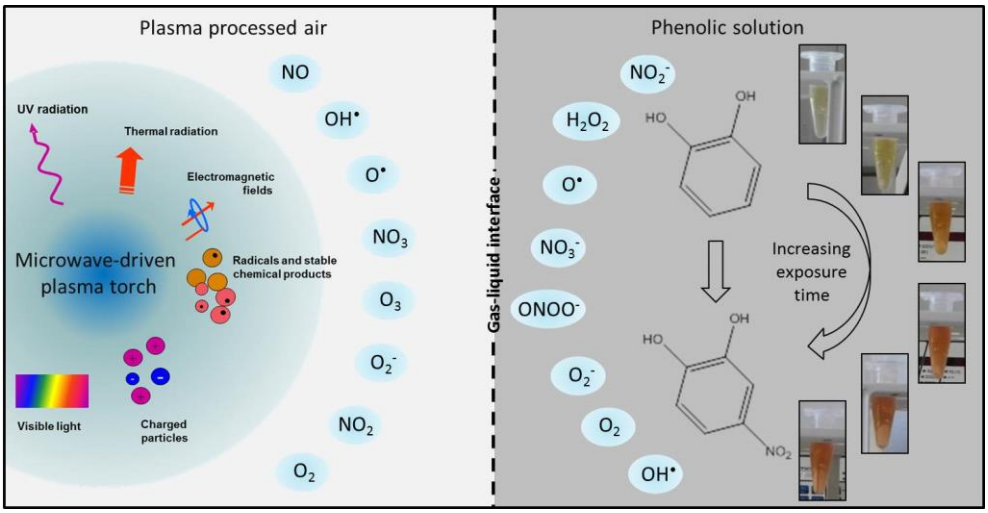

Figure 14: PPA processing for the plasma-induced generation of nitro-phenolic compounds in phenolic model solutions.

The initiation of novel reaction channels might lead to new transient and end products, which is an often highly desired and already exploited result of plasma treatment. However, the generation of high chemically active species harbors the risk of not only uncontrollable, but also undesired plasma-chemical synthesis. As a summary of all chemical reactions in different cold plasmas is almost impossible, a thorough knowledge of plasma reaction chemistry therefore is mandatory for any industrial application. When adequately assessed, selected plasma-induced and plasma-accelerated chemical reactions could contribute to the resource efficient production of chemicals being otherwise produced in costly manufacturing processes. For instance, the nitration of aromatic nuclei represents one of the most basic reactions in organic synthesis and is widely used in the pharmaceutical and chemical industries. Shortcomings, such as over-nitration, formation of regioisomers, and generation of impurities due to oxidation are the driving forces for the continuing research on the reaction of impurities due to oxidation have created a large library of nitration reagents and methods.

Plasma treatment of selected phenolic compounds may induce the formation of nitro-phenolics in aqueous solutions (Fig 14).

As formerly mentioned, both aspects can be detrimental for the CAPP treatment of agricultural food produce and need to be investigated in detail in order to optimize relevant process parameters for their prevention or for the development of tailored applications with regard to related scientific issues. Therefore, this part of the thesis aims at

i. investigating the applicability of CAPP utilizing its surface-modifying effects in order to develop a plasma-assisted preparative method for evidencing a radial strain gradient in cuticles from apple fruit (Section 3.1),

ii. examining the capability of using air plasma to induce the formation of nitro-phenolics in aqueous solutions for developing an alternative process to provide key organic intermediates in pharmaceutical and chemical industry (Section 3.2).

3.1 Evidence for a radial strain gradient in apple fruit cuticles

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SHORT COMMUNICATION

Evidence for a radial strain gradient in apple fruit cuticles

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ABSTRACT

Main conclusion
The morphological outer side of the apple fruit cuticle is markedly more strained than the inner side. This strain is released upon wax extraction.
This paper investigates the effect of ablating outer and inner surfaces of isolated cuticular membranes (CM) of mature apple (Malus 9 domestica) fruit using cold atmospheric pressure plasma (CAPP) on the release of strain after extraction of waxes. Strain release was quantified as the decrease in area of CM discs following CAPP treatment and subsequent solvent extraction of wax. Increasing duration of CAPP treatment proportionally decreased CM mass per unit area. There was no difference in mass loss rate between CAPP treatments of outer or inner surfaces. Also, there was no difference in surface area of CMs before and after CAPP treatment. However, upon subsequent wax extraction, surface area of CMs decreased indicating the release of strain. Increasing the duration of CAPP treatment resulted in increasing strain release up to 47.7 ± 8.0 % at 20 min when CAPP was applied to the inner surface. In contrast, strain release was independent of CAPP duration averaging about 12.1 ± 0.6 % when applied to the outer surface of the CM. Our results provide evidence for a marked gradient of strain between the outer side (strained) and the inner side of the CM (not strained) of mature apple fruit.

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Keywords
Cuticle - Fracture - Microcrack - Stress - Rheology

Abbreviations

A	Surface area
CAPP	Cold atmospheric pressure plasma
CM	Cuticular membrane
CP	Cuticular proper
CL	Cuticular layer
e	Strain

Introduction

The fruit of most species are subject to continuous extension growth throughout a development period of many weeks. Compared with other plant organs this is unusual. Thus, in other determinate organs, such as leaves, the phase of rapid expansion is limited to their early development and this lasts perhaps for 2 or 3 weeks. In indeterminate organs such as shoots and roots, the period of extension for a particular cohort of cells (in the extension zone) is similarly brief. The lengthy period of extension just alluded to applies to apple fruit, where their growth follows a sigmoidal pattern of extension over a period of perhaps 20 weeks (Knoche *et al.* 2011). After an initial lag phase with little change in mass, the rate of fruit volume increase, and hence of surface area increase, rises rapidly. It reaches a maximum value around the middle of the season before decreasing again towards maturity. During the period of maximum increase, growth rates of up to 3 g day-1 (mass) and 2 cm2 day-1 (surface area) have been measured (Khanal,

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unpublished data). The increase in the volume of an apple fruit is caused principally by expansion of the parenchyma of its fleshy mesocarp. This subjects the skin to marked tangential strain and hence, to stress (Skene 1980). The epidermal and hypodermal tissues release the buildup of growth-induced stresses by straining (growing) involving both cell division and cell extension (Skene 1966). The real challenge to maintaining dermal structural integrity is faced by the outermost layer of the skin, the cuticular membrane (CM). The CM is a non-living polymer deposited on the outer cell walls of the epidermal cells (Heredia 2003). It serves as a barrier to transpiration, water uptake and pathogen defence (Köller 1991; Kerstiens 1996; Riederer and Schreiber 2001). Unlike the cellular layers, the CM must at all stages cope with the increase in fruit surface area by straining (stretching). Because CM failure impairs its barrier function, failure is of major concern to the functional integrity of the fruit skin, also to invasion by pathogens and, for commercial fruit producers, it impairs quality. Failure of the CM in apple is usually expressed in the appearance of cuticular microcracks (Faust and Shear 1972a, b). These greatly increase the rate of postharvest water loss (Maguire *et al.* 1999) with increased loss of packed weight during shipping and storage, loss of sheen and, eventually, shrivel. Cuticular microcracking is also the first event preceding the appearance of russeting (Faust and Shear 1972a). This discolouration and roughening of the skin can render fruit less visually attractive, and in some cultivars even unmarketable (Faust and Shear 1972a, b).

The apple fruit CM copes with expansion by the continuous deposition of cuticle such that the mass per unit area of cutin and wax and hence, CM thickness actually increases throughout development (Knoche *et al.* 2011). In addition, apple CM contains large amounts of wax per unit area and wax deposition in the expanding cutin network imposes limits on (elastic) strain and hence, results in stress buildup (Khanal *et al.* 2013). However, upon extraction of wax from CM samples in the laboratory, the elastic strain of the polymer network is released and the CM shrinks (Khanal *et al.* 2013). Because of the sequential deposition of the CM during the course of development, it is inferred that those portions of the CM that were deposited earliest, when fruit was young, must undergo larger increases in surface area compared with those deposited later as the fruit neared its final size at maturity. Hence, we hypothesise (1) that a CM layer deposited when a fruit is young, should, by fruit maturity, be more strained and, thus, should release more elastic strain upon wax extraction, than a CM deposited later on. Next, if it is assumed that CM deposition occurs preferentially on the inner surface of a CM (Heide-Jorgensen 1991; Jeffree 1996, 2006), then we may further hypothesise (2) that a gradient in elastic strain will exist between the older (outer) more-strained CM layers and the younger (inner) less-strained CM layers. Together these hypotheses predict that there will be a radial gradient of stored elastic strain through a CM in the anticlinal direction with greater values being found towards the outside and smaller to the inside. A literature search finds that experimental evidence for such a strain gradient is lacking.

Cold atmospheric pressure plasma (CAPP) treatment allows to chemically and physically modify surface characteristics of polymeric materials without affecting their bulk properties (Fricke *et al.* 2011). Extended CAPP treatment results in the ablation of surfaces. CAPP is often generated from pure argon or mixtures of argon and oxygen.

The main mechanisms of ablating surfaces are ion bombardment and production of chemically active species that oxidise organic compounds. Because of the high etch rate, argon and oxygen plasmas are particularly useful in ablating polymers (Fricke *et al.* 2011). It is important to note that the ablation occurs at atmospheric pressure without significant heating. The number of reports on plasma treatment of

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plant surfaces is limited and different plasma sources were used in the studies (Grzegorzewski *et al.* 2010). Typical biological applications of CAPP include the inactivation, and sterilization of microorganism and the bio-decontamination of surfaces (Foest *et al.* 2006; Moreau *et al.* 2008; Baier *et al.* 2014). For reviews on technical applications, the reader is referred to Tendero *et al.* (2006) and Morent *et al.* (2008).

The objective of our study was to establish the presence/absence of a radial gradient in stored elastic strain in isolated fruit cuticles, also to determine the direction of the gradient, if such should exist. We used the apple fruit CM as a model for this work because of our familiarity with it and because of the high amount of wax per unit area and the marked release of strain upon wax extraction (Khanal *et al.* 2013).

Materials and methods

Plant material and cuticle isolation

Apple fruit (Malus 9 domestica Borkh. cv. Idared) were obtained locally at commercial maturity. Epidermal discs were excised from the equatorial region of the fruit using a cork borer (12 mm diameter). The regions were selected for freedom from visual defects and minimal curvature. Epidermal discs were incubated in an isolation medium containing pectinase [90 ml l-1 (Panzym Super E flüssig; Novozymes A/S, Bagsvaerd, Denmark)] and cellulose [5 ml l-1 (Cellubrix L.; Novozymes A/S); (Orgell 1955)], mM sodium azide (NaN3) prepared in a 50 mM citric acid buffer solution at pH 4.0. The enzyme solution was refreshed periodically until CMs separated from adhering tissue. Subsequently, the cuticles were rinsed in deionized water. A square pattern of four holes (ca. 2.4 mm 92.4 mm,hole diameter 0.5 mm) was punched into the hydrated CM discs using a custom-built punch equipped with four needles. The CM discs were then photographed (91.0) under a dissecting microscope (MZ10F, Leica Microsysteme, Wetzlar, Germany; camera DP71, Olympus; Software Cell^P, Olympus), dried, and weighed on a microbalance (CPA2P; Sartorius, Göttingen, Germany).

From the weight of the CM disc and the cross-sectional area of the cork borer the mass per unit area of the CM was calculated. Thereafter, CM discs were subjected to CAPP treatment. The CAPP allows to physically ablate the surface of the CM on its inner or outer side depending on which side is exposed to the plasma jet. This ablation occurs without significant heating at atmospheric pressure.

Cold atmospheric pressure plasma treatment

The CAPP was generated using an 8 W plasma jet (kINPen 09; Neoplas tools, Greifswald, Germany; Weltmann *et al.* 2009) flushed with a mixture of argon 5.0 (purity [99.999 %) and 0.1 % oxygen (Air Liquide, Düsseldorf, Germany) at a flow rate of 5.4 l min-1 (at ambient temperature and pressure, Multi Gas Controller 647C; MKS Instruments, Andover, MA, USA). When applying a high frequency voltage (1.1 MHz; 2–6 kV peak-to-peak voltage), the CAPP is generated at the tip of the electrode. The power supply was operated at a voltage of 65 V and a resonance balancing of 0.05 A. The shape and temperature of the plasma depends on the gas flow rate, electrical settings of operating parameters and distance to the CM surface. Initial experiments demonstrated that the maximum temperature at the surface of the apple CM when starting the plasma jet increased within 1 min from ambient temperature to about 38 _C (ThermoScan 500; Flir, Frankfurt, Germany), but slowly thereafter (rate 0.097 _C min-1). After 30 min, the maximum temperature did not exceed 41 °C (Bußler, unpublished data). Within this range (24–41 °C) temperature has essentially no effect on the rheological properties of the apple fruit CM (Khanal *et al.* 2013). For treating CMs, the plasma jet was fixed in a stage clamp. CM discs were mounted flat between two paper discs. The upper disc had a 10 mm diameter hole in

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the centre that served as an aperture for the plasma. The paper/ CM 'sandwich' was positioned on a holder, placed on a lab jack and aligned underneath the jet. The distance between the plasma jet and CM surface was 8 mm. This setup and geometry allowed an area of about 8 mm diameter in the centre of the CM disc to be subjected to CAPP treatment for durations of 5, 10, 15, 20 min and, where possible, for 30 min. Untreated CM discs served as controls.

Scanning electron microscopy (SEM)
Outer and inner surfaces and cross sections of CM and of dewaxed CM (DCM) with and without CAPP treatment were viewed in a Quanta 200 SEM (FEI Europe Main Office, Eindhoven, The Netherlands). Cross sections were obtained by freeze fracturing in liquid N2. Specimens were mounted on aluminium stubs using conducting carbon tape. Cross sections were viewed at 91,000 and an acceleration potential of 10 kV, the surfaces at 93,000 and 910,000 using an acceleration potential of 20 kV. The time between CAPP treatment and SEM did not exceed 24 h. During this period CMs were held at ≈0 °C above dry silica gel.

Determination of mass loss and biaxial strain release of CM after CAPP
Following CAPP treatment CM discs were recut to 6 mm diameter using a biopsy punch (pfm Medical, Kai Industries Co., Seki City, Japan) such that the 6 mm disc enclosed the centre of the CAPP-treated area and the square pattern of holes. To measure mass loss after CAPP treatment, the CM discs were weighed and their masses per unit area calculated as described above. The mass loss was calculated from the mass per unit area before and after CAPP treatment. Wax mass and strain release of CAPP treated and untreated discs were quantified using the procedures described by Khanal *et al.* (2013). Briefly, the CAPP-treated discs were hydrated in deionised water at 22 _C for 16 h, photographed, redried and soxhlet extracted for 2.5 h using chloroform/methanol (1:1, v/v) to remove wax. The DCMs were dried, reweighed to quantify the mass per unit area, then rehydrated in deionised water at 22 °C for 16 h and rephotographed. The number of individual disc replicates ranged from 5 to 11. For strain assessment, the areas enclosed by the square pattern of holes on the CM disc before (ACM) and after CAPP treatment (ACAPP CM) and after subsequent wax extraction (ACAPP DCM) were quantified on calibrated images by image analysis (software package Cell^P; Olympus Europa). Because there was essentially no change in area of CM discs following CAPP treatment only
(-0.9 ± 0.2 %), analysis was limited to the strain released after CAPP treatment and subsequent wax extraction. The percentage strain (e) released was calculated from the equation

$$\varepsilon = \frac{A_{CM} - A_{DCM}^{CAPP}}{A_{DCM}^{CAPP}} \, x \, 100$$

Statistics
Data are presented as means ± standard errors of the means (SE). Where error bars are not shown they were smaller than the symbols. Regression analysis was performed using SAS (version 9.1.3; SAS Institute, Cary, NC, USA).

Results

The outer surface of isolated apple fruit CM was covered with numerous wax crystals that were distributed uniformly over the surface (Fig. 1a, g). Following CAPP treatment of the outer CM surface, the number of small, angular, wax deposits decreased and fewer larger deposits with rounded edges appeared (Fig. 1b, h). Solvent extraction removed all wax deposits (Fig. 1c). The cutin matrix above the major anticlinal cell walls appeared to be slightly depressed. The inner surface of apple CMs revealed extensive pegging in anticlinal regions of groups of epidermal cells and—within these groups on a smaller scale—between individual cells (Fig. 1d). There was little difference in pegging

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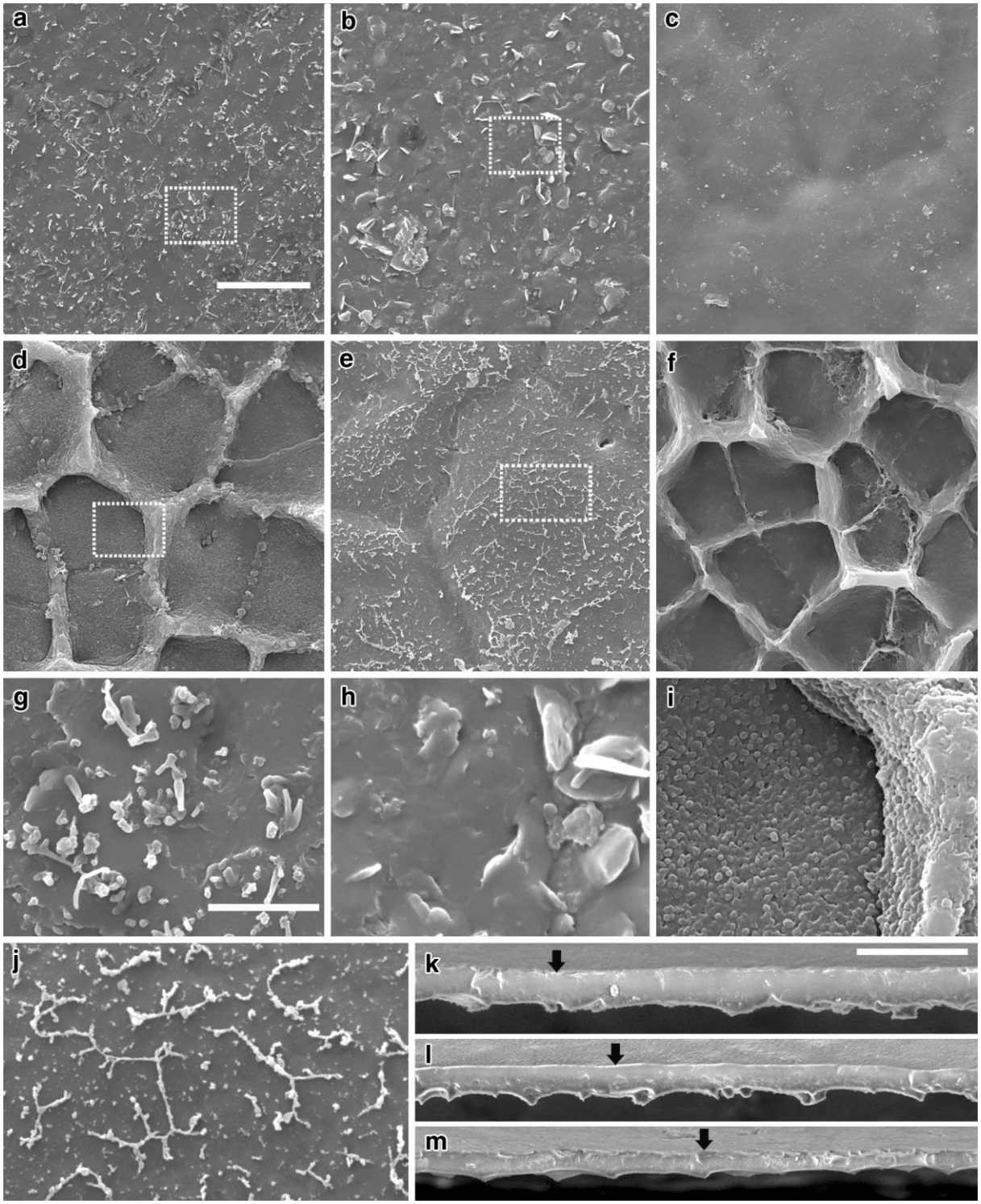

between non- extracted and extracted CMs (Fig. 1d, f).

Fig. 1 Scanning electron micrographs of morphological outer surface (a–c, g, h) and inner surface (d–f, i, j) of 'Idared' apple fruit cuticular membrane (CM; a, d, g, i), cold atmospheric pressure plasma (CAPP) treated CM (b, e, h, j), and dewaxed CM (c, f). CMs were ablated by applying CAPP on the morphological outer surface (b, h) or innersurface (e, j). g, h, i, and j Magnified view (910,000) of the area enclosed by white dotted boxes in a, b, d, and e, respectively. Cross sections obtained by freeze fracture of the CM (k), CM treated with CAPP on the outer surface (l), or on the inner surface (m). Scale bars 20 lm (a–f; 93,000), 5 lm (g–j; 910,000), 50 lm (k–m; 91,000).

However, CAPP treatment of the inner surface of a CM essentially removed all pegs, leaving behind a smooth, less-sculptured surface with slight depressions

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in regions of the former pegs (Fig. 1e). Cross sections revealed a continuous cuticle in ‘Idared’ apple of fairly uniform thickness (Fig. 1k). Unlike apple fruit CMs of other cultivars, epidermal cells were not encased by the CM.

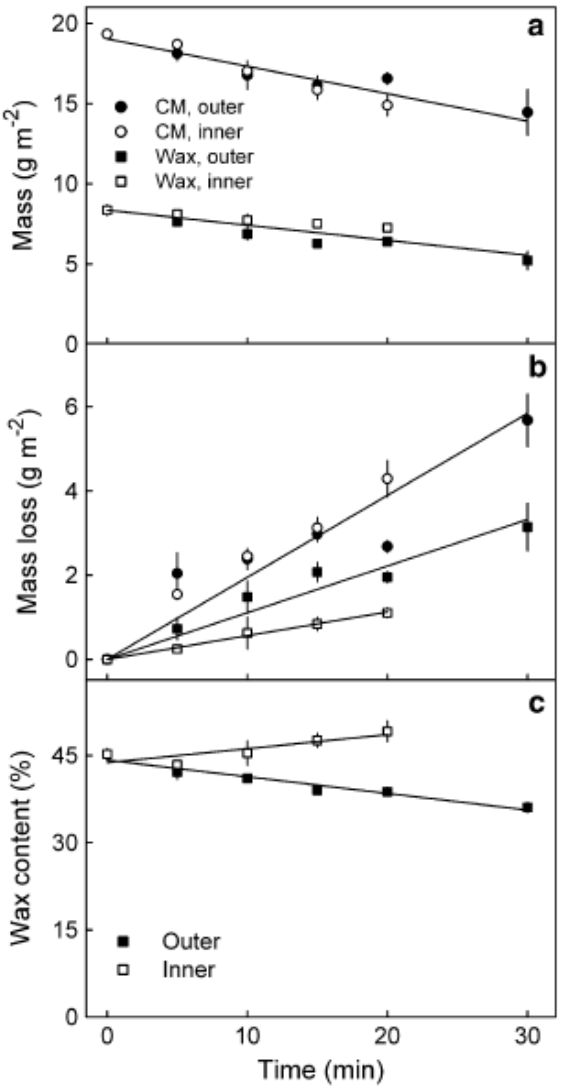

Fig. 2 Effect of the duration of cold atmospheric pressure plasma (CAPP) treatment of the morphological inner (inner) or morphological outer surfaces (outer) of cuticular membranes (CM) isolated from mature ‘Idared’ apple fruit (Malus 9 domestica) on the CM and wax mass per unit area (a), the loss in CM and wax mass per unit area (b), and the wax content of the CM (c). Data points represent mean ± SE, n = 5–9 (treated) and 11 (untreated).

Treating CMs with CAPP on the outer surface decreased CM thickness (Fig. 1l). When applied to the inner surface, the pegs disappeared and CM thickness decreased (Fig. 1m). Increasing the duration of CAPP treatment, resulted in a linear decrease in CM mass and wax mass per unit area (Fig. 2a). CAPP treatment of the inner surface beyond 30 min or of the outer surface beyond 45 min resulted in the formation of holes. There was essentially no difference in the decrease in CM mass when the outer or inner surfaces were treated for up to 15 min. However, the decrease in wax mass was larger when a CM was treated from the outer surface than from the inner one (Fig. 2b). The percentage wax increased slightly when inner surfaces of CM were treated with CAPP, but decreased consistently when the outer surfaces were treated (Fig. 2c).

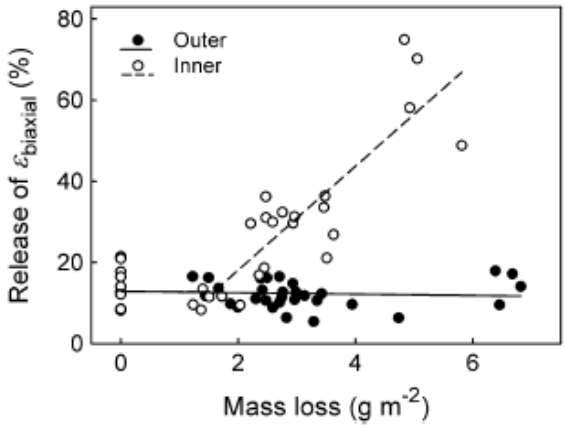

Fig. 3 Effect of mass loss due to cold atmospheric pressure plasma (CAPP) treatment of the morphological inner (inner) or outer (outer) side of the cuticular membrane (CM) of ‘Idared’ apple (Malus 9 domestica) fruit on the release of biaxial strain after extraction of wax. The release of biaxial strain was measured by quantifying the shrinkage of discs of CM after CAPP treatment and subsequent wax extraction. For details see ‘Materials and methods’.

Extracting wax from CM significantly decreased the area of CM discs indicating the release of stored elastic strain. When CAPP was applied to the inner surface of the CM, a biphasic relationship between mass loss caused by CAPP and the resulting strain release was obtained. There was no effect of CAPP treatment and subsequent wax extraction on strain release up to a mass loss of the CM of ≈1.6 g m-2 (Fig. 3). Above this threshold, the release of strain following CAPP treatment and wax extraction was positively and linearly

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related to the loss of CM mass per unit area due to CAPP (R^2 = 0.76, P\0.0001; Fig. 3). In contrast, when CAPP was applied to the outer surface, strain release after wax extraction was low and independent of the loss of CM mass per unit area (R2 = 0.005, P = 0.72; Fig. 3).

Discussion

Our results demonstrate that CAPP treatment of the morphological inner surface of CM, but not of its outer surface, results in elastic strain release upon wax extraction. When CAPP is applied to the inner surface of the CM, the strain release on wax extraction increases linearly with increasing duration of CAPP treatment and thus with increasing CM mass loss. In contrast, when CAPP is applied to the outer surface of the CM, strain release is independent of treatment duration. The simplest, and therefore most plausible, explanation for the observed asymmetry in strain release is that there is a gradient in stored elastic strain across the CM (from the point of view of the whole apple, the gradient lies in a radial direction). Here, the outer layers of the CM (but not the inner ones) are strained elastically. This elastic strain has been fixed within the CM polymer by the subsequent deposition of wax (Khanal *et al.* 2013). This inference is consistent with several findings reported in the literature. First, cuticles often have a bilayer structure where the outer layer is referred to as the cuticle proper (CP) and the inner layer as the cuticular layer (CL; for reviews see Jeffree 1996, 2006). The CP appears very early during development, whereas the CL is deposited later at the cell wall cuticle interface (Heide-Jorgensen 1991; Jeffree 2006). Thus, as the oldest layer the CP undergoes the largest area expansions. For example, the fruit undergoes a 50-fold increase in surface area from a 10 mm diameter fruitlet to a 70 mm diameter mature apple. In addition, the maximum relative growth rates in surface area typically occur during the first weeks after full bloom and thus, the CP is also subjected to the highest relative growth rates (Wertheim 1982; Knoche *et al.* 2011). In contrast to the CP, the CL at the inner side of the CP forms during later stages and, therefore, will be less strained than the CP. Since cutin and wax deposition parallels fruit growth in apple, the wax deposited in the expanding cutin matrix fixed the strain of CP and CL (Khanal *et al.* 2013). The difference in strain history between CP and CL caused more strain to be released after wax extraction from the outer CP than the inner CL. This interpretation is consistent with the bilayer fine structure of the CM. Conclusive experimental evidence, however, on an hypothetical gradient in deposition and polymerisation of cutin is lacking. Second, a larger strain at the physiological outer side of the CM is also consistent with the characteristic microcracks frequently observed on the surface of apple fruit. These cracks are often limited to the outer (and—based on our data—more strained) layer of the CM (Maguire *et al.* 1999; Roy *et al.* 1999; Curry 2009). Third, CM often roll up and curl upon isolation with the morphological outer surface being located inside the curl. Finally, a radial gradient in strain has been reported for the skin composite of sweet cherry fruit where the CM as the outer most layer is more strained than the underlying epi- and hypodermis (Knoche and Peschel 2006; Grimm *et al.* 2012). Differential strain fixation of the inner layer of the CM by cuticle constituents that are not solvent extractable is equally unlikely. Structural carbohydrates such as cellulose are one such constituent of enzymatically isolated CM and—when encrusted with cutin—may not be accessible to the enzymatic isolation medium (Schreiber and Schönherr 1990). However, embedded cellulose is unlikely to be responsible for the gradient in strain of the CM. First, fluorescence light microscopy and calcofluor white staining revealed that the amount of cellulose located in the inner layer of the apple fruit CM was small (Khanal, unpublished data). Second, the CM of ‘Idared’ apple has essentially no hypodermal development and

hence, epidermal and hypodermal cells and their cell walls are not encased in the cutin matrix as often observed in apple fruit CMs of other cultivars. Thus, we would expect most cellulose of the cell wall to be accessible to the isolation enzymes. Third, a 5 min CAPP treatment of the inner side decreased CM mass by about 8 %, removed essentially all cellulose from the inner side as indexed by the lack of calcofluor white fluorescence in fluorescence microscopy (Khanal, unpublished data), yet had no effect on strain release as compared to the non-CAPP-treated control or CMs that were subjected to extended CAPP treatments of the inner side. It may be argued that the application of CAPP produced other effects in addition to the ablation of CM material. Such effects may include the introduction of oxygen-containing functional groups and the creation of new polymer cross-links at the surface of the remaining material (Fricke *et al.* 2011). At present, we do not have any indication that this was a factor with the weak plasma jet used in our study. First, the cuticle of mature apple fruit is a continuous, non-stomatous polymer film that presents a significant penetration barrier also for gas molecules and - particularly so - for ionised molecules. Also, CAPP treatment of polymers is reported to modify properties of the surface, but not those of the bulk of the polymer (Fricke *et al.* 2011). Both arguments would make any effects on subsurface properties of the CM such as the strain relaxation after wax extraction unlikely. Second, if any modifications of the CM would have happened, these would occur regardless of the orientation of the CM relative to the plasma jet. Thus, none of these effects would account for the differential strain relaxation observed in our study.

In summary, the results presented provide direct evidence for a gradient in strain in the cuticle of apple and possibly other fruit. The data are consistent with the view that the older and morphologically outer regions of the CM that are deposited during early fruit development are subjected to larger strains than the more recently deposited younger regions at the cuticle/cell wall interface. This strain is ‘‘fixed’’ by the deposition of wax and released upon wax extraction indicating that this strain is reversible.

Author contribution

MK and OS initiated the study.
BPK, MK, SB and OS designed the experiments.
BPK and SB performed the experiments.
BPK and MK analysed the data and wrote the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

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3.2 Plasma processed air as an innovative approach for the synthesis of nitrophenolic compounds

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Plasma processed air as an innovative approach for the synthesis of nitrophenolic compounds

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ABSTRACT

Nitration of aromatic nuclei is one of the most basic reactions in organic synthesis and is widely used in the pharmaceutical and chemical industries. Shortcomings, such as over-nitration, formation of regioisomers, and generation of impurities due to oxidation are the driving forces for the continuing research on the reaction that has created a large library of nitration reagents and methods. Aim of this study was to investigate the capability of using a microwave-driven (MW-driven) plasma discharge to induce the formation of nitro-phenolics in aqueous solutions.
For this purpose, model phenolics (pyrocatechol) as well as characterized derivatives of hydroxycinnimic acid (chlorogenic acid and caffeic acid) were selected in order to specify the reaction products generated by exposure of the liquid systems to plasma processed air (PPA). Thereby, the hypothesis was tested that plasma treatment with air as production gas can cause a nitration of aromatics. Antioxidant capacity, pH value and UV/Vis spectra were used as exemplary markers providing information on the changes caused by the plasma treatment. Special emphasis was laid on developing methods for separation and identification of reaction products based on reverse phase high performance liquid chromatography (RP-HPLC) and liquid chromatography/mass spectrometry (LC/MS) in order to gain first insights into the occurring reaction mechanisms and to identify nitrated reaction products formed.
Exposure to PPA caused a perceptible color change towards yellow-brown accompanied by a strong reduction of the pH and the formation of insoluble sediments in the model solutions. The accumulation of nitrate, nitrite and hydrogen peroxide was evidenced. MS analysis demonstrated the formation of aromatic nitro-phenol derivatives in all tested systems. The main reactions observed when the liquid model solutions were exposed to PPA were attributed to oxidation, nitration and polymerization of the phenolic compounds.

1. Introduction

Nitration of aromatic compounds is one of the most fundamental reactions in organic synthesis, which provides key organic intermediates or energetic materials and therefore is an immensely important process in pharmaceutical and chemical industry (Olah, Malhotra & Narang, 1989). Aromatic nitro compounds represent versatile intermediates for a wide range of industrial products, like pharmaceuticals, dyestuffs and explosives. Traditionally, nitration of aromatic compounds respectively the introduction of a nitro group into an aromatic ring is commonly performed in strongly acidic polar media as a mixture of nitric and sulfuric acids (Schofield, 1980; Olah *et al.*, 1989). However, the problems associated with this method, which is often deteriorated with poor selectivity for desired products and high environmental cost, have prompted the search for alternative methods. As phenols are highly reactive, the nitration of phenols by mixed acids is always associated with the formation of dinitro compounds, oxidized products, and unspecified resinous materials. A plethora of new procedures have become available recently which address contemporary issues such as selectivity, safety, economy, waste and the environment (Olah *et al.*, 1989) and a lot of mild nitration processes for phenols have been developed to overcome these shortcomings. Especially, in recent years, various nitrate salts for phenols have been reported (Suzuki *et al.*, 1982; Alif & Boule, 1991; Fischer & Warneck, 1996; Torrents *et al.*, 1997; Mulvaney, Wagenbach & Wolff, 1998; Honrath et al., 1999; Jones *et al.*, 2000; Honrath *et al.*, 2002; Vione *et al.*, 2003). Various clean

nitration approaches have been explored involving nitrogen oxides such as NO_2 (Sato & Hirose, 1998; Peng & Suzuki, 2001), N_2O_4 (Iranpoor, Firouzabadi & Heydari, 2003; Zolfigol *et al.*, 2008), and N_2O_5 prepared by the oxidation of N_2O_4 with O_3 (Bakke *et al.*, 1994) or recyclable catalysts such as lanthanide triflates (Waller *et al.*, 1997), perfluorinated resin immobilized sulfonic acid (Cheng *et al.*, 2008), claycop or zeolites (Gigante *et al.*, 1995; Choudary *et al.*, 2000; Smith, Almeer & Peters, 2001). However, some of the nitrating reagents are poorly regioselective and uneconomical. Considering these concerns, there is still a good scope for research towards finding economic, mild reagents for regioselective nitration of phenols.

It is well known that nitrite/nitrate photolysis in natural waters can trigger hydroxylation, nitration, and nitrosation reactions of many organic compounds (Matykiewiczová *et al.*, 2007). The photochemistry of nitrite irradiated in the range of 250–400 nm is known to be very complex and it essentially results in the formation of NO• and $O^{\bullet-}$ (Eq. (1)), whereas $O^{\bullet-}$ can be protonated to form HO• (Eq. (2)). Further, NO_2^- and NO• can be oxidized by HO• or by dissolved oxygen, respectively, to form NO_2• (Eqs. (3) and (4)).

$$NO_2^- + h\nu \rightarrow NO^\bullet + O^{\bullet-} \quad (1)$$

$$O^{\bullet-} + (H^+) \rightarrow HO^\bullet + HO^- \quad (2)$$

$$NO_2^- + HO^\bullet \rightarrow NO_2^\bullet + HO^- \quad (3)$$

$$2NO^\bullet + O_2 \rightarrow 2NO_2^\bullet \quad (4)$$

In aerated aqueous solutions the nitrate photochemistry results in two main processes, shown in Eqs. (5) and (6), The former, producing reactive hydroxyl radical and nitrogen dioxide, requires a source of the proton (Mack & Bolton, 1999).

$$NO_3^- + h\nu \rightarrow NO_2^\bullet + O^\bullet \; H_2O^- \rightarrow NO_2^\bullet + HO^\bullet + HO^- \quad (5)$$

$$NO_3^- + h\nu \rightarrow NO_2^- + O^\bullet \quad (6)$$

The excitation of nitrite or nitrate ions may induce oxidation, nitration or nitrosation on phenolic derivatives resulting from the direct excitation of aromatic compounds in the presence of nitrate ions (Suzuki *et al.*, 1982) or from the excitation of nitrate ions (Mulvaney *et al.*, 1998; Jones *et al.*, 2000). From investigations concerning the presence of oxygen it is concluded the competition of two mechanisms, one involving N_20_3 and the other the radical adduct phenol-NO_2•. With pyrocatechol nitration is favored by deoxygenation. In environmental conditions nitration or nitrosation by excitation of NO_2^- are highly unlikely. For the industrial production of nitrated phenolic compounds several approaches were pursued in order to trigger the aforementioned specific reactions. An up to now absolutely disregarded possibility for the targeted induction of desired reactions is the use of nonthermal atmospheric pressure plasma as a well-known source of highly reactive species. Plasma is described as a neutral ionized gas comprising particles such as photons, electrons, positive and negative ions, atoms, free radicals, and excited and non-excited molecules, which are in permanent interaction. Therefore, plasma has the potential for its reactive components to induce a multitude of interactions and chemical reactions with organic compounds.

Aim of this study was to investigate the capability of using a microwave-driven (MW-driven) plasma discharge to induce the formation of nitro-phenolics in aqueous solutions. MW-driven discharges are generated without electrodes meanwhile the microwaves are generated by a magnetron (typically at 2.45 GHz) and are guided to the process chamber by a wave guide or a coaxial cable. The absorption of the microwaves by the electrons present in the process gas leads to an increase in kinetic energy and thus ionization reactions by inelastic collisions (Ehlbeck *et al.*, 2011). The major advantage of MW-driven discharges is that they can be ignited in air environment. The gas consumption is in a moderate range of some slm (standard liter per minute). Depending on the discharge gas used, a high amount of reactive species can be produced (e.g., N_xO_y, O_3, O_2, and OH) (Uhm, Hong & Shin, 2006). In the context of gas–liquid interfaces and with regard to the formation of nitrophenolic compounds, reactive nitrogen species (RNS) generated by the use of air as the process gas are further of special interest. Indirect or remote plasmas are usually generated in a separated chamber. Consequently, only the plasma processed air (PPA) containing longer-living reactive species such as nitric oxide or ozone comes into contact with the liquid to be treated. At the liquid-gas interface the excitation of nitrite or nitrate ions may induce oxidation, nitration or nitrosation on phenolic derivatives resulting from the direct excitation of aromatic compounds in the presence of nitrate ions (Suzuki *et al.*, 1982) by plasma-immanent species or from the excitation of nitrate ions (Mulvaney *et al.*, 1998; Jones *et al.*, 2000), which are formed in the liquid.

In this study, model phenolics (pyrocatechol) as well as characterized derivatives of hydroxycinnimic acid (chlorogenic acid and caffeic acid) were selected in order to specify the reaction products generated by exposure of the liquid systems to PPA. Thereby, the hypothesis was tested that plasma treatment with air as production gas can cause a nitration of aromatics. Antioxidant capacity, pH value and UV/Vis spectrum were used as exemplary markers providing information on the changes caused by the plasma treatment. Special emphasis was set on developing methods for separation and identification of reaction products based on reverse phase high performance liquid chromatography (RP-HPLC) and liquid chromatography/mass spectrometry (LC/MS) in order to gain first insights into the occurring reaction mechanisms and to identify plasma-induced nitrated reaction products.

2. Material and Methods

2.1 Sample preparation

In order to gain information on the effect of different substituents at the aromatic ring on possible oxidation, nitration or polymerization reactions, three model compounds served as test material: pyrocatechol (benzene-1,2-diol, Carl Roth, Karlsruhe, Germany), chlorogenic acid ((1S,3R,4R,5R)-3-[(E)-3-(3,4 dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid, Sigma Aldrich, Steinheim, Germany) and caffeic acid ((E)-3-(3,4-dihydroxyphenyl)prop-2-enoic acid, Sigma Aldrich, Steinheim, Germany).

The hydroxycinnimic acid derivates used were solved in distilled water and each diluted to a final concentration of 10 mM. Following exposure to PPA the solutions were frozen in liquid nitrogen in aliquots of 2 mL and kept at -80°C until further analysis. Due to the plasma-induced formation of insoluble polymers the samples were vortexed, sonicated for 10 min and centrifuged (10000 g, 5 °C, 5 min) twice following thawing at ambient temperature. Clear supernatants were used for further analysis.

2.2 Exposure to plasma processed air (PPA)

Exposure to PPA was conducted using a microwave-driven plasma setup (PLexc®, INP, Greifswald, Germany). Air with

a gas flow of 18 slm (standard liter per minute, 30.3975 (Pa*m^3)/s) was used as the process gas. At a frequency of 2.45 GHz and a power consumption of 1.2 kW the microwave generated plasma process gas (burst mode with an ignition/pause-cycle of 20x5s/7s) had a peak temperature of about 3700 °C. PPA was cooled down to 22 °C by flowing through a water-cooled (4°C) double-lined pipe located directly behind the microwave driven plasma torch. Pipa *et al.* (2012) characterized the microwave plasma used via optical emission spectroscopy. Further details regarding the plasma source set-up can be found elsewhere (Schnabel *et al.*, 2015). Exposure to PPA was carried out in a reaction chamber with a volume of about 30 L. Each 10 mL of the respective sample solution were transferred into a glass petri dish and put into the reaction chamber, which was filled with PPA letting it take effect for 1, 2.5, 5, 7.5 or 10 min, respectively. PPA treatment was terminated by ventilation of the reaction chamber with air. Trials were carried out in triplicate and in randomized order.

2.3 pH measurement and UV/Vis spectroscopy

Prior to and following PPA treatment, the pH of the solutions was measured (S20-SevenEasyTM pH, equipped with an InLab® Micro micro combination pH electrode, Mettler-Toledo, Berlin, Germany). UV/Vis spectra ranging from 190 to 1000 nm of the 1:10 diluted samples were recorded in a double beam spectrophotometer (Specord® 50 Plus, Analytik Jena, Jena, Germany) against a pure water sample in high quality quartz cells with a 1-cm optical path length.

2.4 Quantification of nitrite, nitrate and hydrogen peroxide accumulation

In order to gain deeper insights into possible plasma-induced reactions at the liquid-gas interface the formation of NO_2^-, NO_3^- and H_2O_2 was analyzed. To avoid interactions between plasma-immanent species and other components except water, pure autoclaved water was used as test material. As nitrite ions react with sulfanilic acid to form a diazonium salt in acidic solution, which in turn reacts with N-(1-naphthyl) ethylenediamine dihydrochloride to form a red-violet azo dye, the color shift was determined photometrically in semi-microcell at 520 nm (DIN EN 26 777). Samples were diluted with pure water to ensure final NO_2-N concentration in the range between 0.2 and 1 mg/L whereas an absorbance of 0.010 A corresponds to 0.004 mg/L NO_2-N. NO_3^- concentration was analyzed analogous to DIN 38405-9. In sulfuric and phosphoric solution nitrate ions react with 2,6-dimethylphenol (DMP) to form 4-nitro-2,6-dimethylphenol that was determined photometrically at 320 nm (DIN 38405-9). Samples were diluted with pure water to ensure final NO_3-N concentration in the range between 1 and 25 mg/L whereas an absorbance of 0.010 A corresponds to 2 mg/L NO_3-N. The concentration of accumulated H_2O_2 was measured spectrophotometrically at 400 nm as hydrogen peroxide reacts with titanyl sulfate ($TiOSO_4$, 6.25 mM in acid solution (H_2SO_4, 8 %) to form a yellow pertitanic acid complex. The assay consisted of 1500 µL sample solution and 125 µL of the reagent, hydrogen peroxide was used for the calibration.

2.5 Trolox Equivalent Antioxidant Capacity (TEAC) assay

The Trolox Equivalent Antioxidant Capacity (TEAC) assay is based on the scavenging of the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical (ABTS•) converting it into a colorless product. The degree of decolorization induced by a compound is related to that induced by trolox giving the TEAC value (Razzaghi-Asl *et al.*, 2013). The TEAC assay described by Re et al. (1999) was used in this study. An ABTS• stock solution was prepared by mixing 700 µM ABTS (Sigma Aldrich, Steinheim, Germany) with 245 mM potassium persulfate in 5 mM phosphate buffer (pH 7.4), containing 150 mM NaCl (PBS). The solution was protected from light and stored at room temperature for 12-16 h. ABTS• working solution was prepared by 1:10 dilution of the ABTS• radical-cation stock solution with PBS buffer. Due to a gradual decrease in absorbance of the ABTS• working solution (ca 2% per hour) appropriate blanks were recorded for each measurement (the blank is the decrease in absorption of the solvent without the compound added). The calculation of TEAC in mM was based on creating a calibration curve with trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Sigma Aldrich, Steinheim, Germany). A 2.5 mM stock solution was diluted to final concentrations ranging from 0 to 0.25 mM in PBS buffer. Despite the strong acidity of the PPA treated hydroxycinnimic acid derivate solutions, a TEAC assay pH of 7.4 could be ensured in order to avoid falsification of the measurement by diluting the acidic samples with PBS buffer to final concentrations of 1mM (pyrocatechol) and 0.1 mM (chlorogenic acid and caffeic acid), respectively. The assay consisted of 10 µL of sample or standard solution and 150 µL of ABTS• working solution. A microplate spectrophotometer (PowerWave HT, BioTek, Bad Friedrichshall, Germany) was used for reading the absorbance at 730 nm following 100 s of shaking, 400 s reaction time and 100 s shaking of the mixtures in 96 well polystyrene plates (Greiner Bio-One, Kremsmuenster, Austria).

2.6 RP-HPLC analysis

An RP-HPLC system consisting of degasser (DG-1310, Sanwa Tsusho, Tokyo, Japan), two single plunger reciprocating pumps (LC-10AS, Shimadzu, Kyoto, Japan), auto injector (SIL-10A, Shimadzu, Kyoto, Japan), column oven (model No. 1250426, Bio-Rad, Hercules, USA) and UV/Vis detector (SPD-10A, Shimadzu, Kyoto, Japan) was used to determine the reaction products. A ProntoSIL® 120-3-C18 ace-EPS (150 mm × 4.6 mm, 3.0 µm, CS-Chromatographie Service GmbH, Langerwehe, Germany) was used to separate the compounds at a 0.8 mL min-1 flow and a temperature of 25°C. Eluent A was 2% acetic acid and eluent B was 100% methanol. Gradient used for eluent B was 0-18% (0-3 min), 18-23% (3-8 min), 23-29% (8-14 min), 38% (14-18 min), 48% (18-22 min), 58% (22-25 min) and 10% (25-30 min).

The analytes were detected at 274 and 319 nm using an injection volume of 10µL in order to ensure a sampling/rinsing speed of 35/5 µL·s^{-1}. Catechol, chlorogenic acid and caffeic acid were used as standards for external calibration curves. Stability of detected analytes was tested by analyzing the samples on three occasions at intervals of 24 h.

2.7 HPLC-MS analysis

An HPLCMS system consisting of an oil-sealed rotary pump (E2M28, BOC Edwards, West Sussex, UK) Edwards, a nitrogen generator (Ecoinert ESP 4, DWT, Essen, Germany) degasser (DGU-20 A5, Shimadzu, Kyoto, Japan), two single plunger reciprocating pumps (LC-20 AS, Shimadzu, Kyoto, Japan), auto injector (SIL-20A, Shimadzu, Kyoto, Japan), column oven (CTO-20 AC, Shimadzu, Kyoto, Japan) and mass spectrometer (LCMS-2010EV, Shimadzu, Kyoto, Japan) in negative mode was used to determine the reaction products. A ProntoSIL® 120-3-C18 ace-EPS (150 mm × 4.6 mm, 3.0 µm, CS-Chromatographie Service GmbH, Langerwehe, Germany) was used to separate the compounds while the separation of analytes was performed exactly as that of the RP-HPLC analysis except for the flow F, which was halved, the gradient times were doubled correspondingly. The

absorbance was recorded from 190 to 370 nm using the D2 lamp of the photodiode array detector (SPD-M20 A, Shimadzu, Kyoto, Japan). Negative ion mass spectra of the samples were recorded in the m/z range of 50–500 m/z, at a scan speed of 500 amu sec-1 (event time 1 sec). Nitrogen was used as drying gas at a flow rate of 1.5 l min-1. The nebulizer temperature was set at 250°C and a potential of 5 KV was used for ionization.
The mass intensity peaks at the retention times previously determined for selected specific products were examined for their m/z values. The applied procedure allowed to determine whether or at what point of the separation the m/z (dimensionless [40]) = M (substance) + 46 – 1 – 1 contributed to the TIC (total ion current) – which would be the m/z of a mononitrated reaction product in negative ionization mode (deprotonated molecule). Obtained chromatograms were analyzed using LCMS solution software (Vers. 3, Shimadzu, Kyoto, Japan). Only the sample solutions exposed to PPA for 10 min were analyzed as detected peaks of the reaction products were found to be most prominent compared to all other treatment times.

2.8 Statistical analysis and modelling of reaction kinetics
All experiments were conducted at least three (or more) times. The number of parallel samples in the experiments was six or more. All data are expressed as means of their standard deviations. The results were analyzed using GraphPad Prism 6 (Vers. 6.01, GraphPad Inc. CA, USA). Unpaired samples were analyzed by the ANOVA one-way analysis of variance using Tukey's multiple comparisons test. If there was a significant difference, pairwise comparison was carried out. Values of $p < 0.05$ were considered statistically significant.
The mean values of the pH and pyrocatechol content data were fitted with GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool), a freeware Add-Inn for Microsoft ® Excel by using a biphasic inactivation model (Cerf, 1977). In this model, the relation between pH and pyrocatechol content, respectively, and exposure time is given by the equation:

$$\frac{y\,(t)}{y_0} = \varphi \cdot e^{k_1 \cdot t} + (1 - \varphi) \cdot e^{k_2 \cdot t} \qquad (7)$$

y(t) is the measured pH or pyrocatechol content after a certain plasma exposure time (t) and y_0 is the initial pH or pyrocatechol content. φ is a constant designating the transition from the first reaction phase to the second whereas k_1 and k_2 represent corresponding rate constants.

3. Results and Discussion

The intention of this study was to provide evidence for the formation of aromatic nitro-phenol derivatives upon exposure of different hydroxycinnimics acid to PPA. It was further envisaged to gain information on the effect of different substituents at the aromatic ring on possible oxidation, nitration or polymerization reactions and to elucidate the stability of such adducts. First indications for the applicability of PPA as an efficient and facile nitration procedure for phenols are reported.

3.1 Impact on pH of the model solutions
Exposure to PPA caused a decrease in pH in all sample solutions treated (Fig. 1A-D). The most rapid and steep deterioration of the pH was detected in pure water characterized by a drop in pH from 6.0 to 1.5 (ΔpH 4.5). Regarding the pyrocatechol solution the pH dropped from 5.3 to 3.1 (ΔpH 2.2) within the first minute of exposure to PPA. Decrease of pH was less pronounced in caffeic acid (ΔpH 0.8) and even less for chlorogenic acid (ΔpH 0.5) solution. Extending the treatment time lead to the progressive decrease of the pH in all sample solutions resulting in final reductions of 2.9 (catechol), 1.5 (caffeic acid) and 1.0 (chlorogenic acid), respectively. Further, all pH kinetics parameters show an accelerated initial decrease further leveling off in a tailing for longer treatment times. This plasma-induced behavior of the pH could be adequately described with a biphasic reaction model. The root mean sum of squared error (RMSE) values for each fit (Table 1) indicate that the biphasic model was well suited to represent the experimental data.

Table 1 Means (± sd; n = 6) of reaction rate constants k_1 and k_2 for a biphasic fit of the pH decrease in pyrocatechol, chlorogenic acid and caffeic adic model solutions and in pure water. Goodness-of-fit is presented via R^2 and RSME values.

	k_1 [-]	k_2 [-]	R^2	RSME
Pure water	-10.14 (± 0.71)	0.00 (± 0.05)	0.9980	0.1266
Pyrocatechol	-6.69 (± 0.20)	-0.16 (± 0.01)	0.9999	0.0135
Chlorogenic acid	-4.45 (± 0.41)	-0.17 (± 0.4)	0.9708	0.1070
Caffeic acid	-3.51 (± 1.18)	-0.17 (± 0.03)	0.9947	0.0634

The general types of chemical reactions occurring at the gas–liquid interface can be separated into acid–base reactions, oxidation reactions caused by ROS and RNS, reduction reactions caused by reductive species (e.g., H and HO radicals) and photochemical reactions initiated by UV radiation from the plasma. Acidic effects can be caused by secondary species formed by plasma, for instance nitrous and nitric acids, excited nitrogen species, and their products (NO_2^-, NO_3^-) as well as by the formation of singlet oxygen (Brisset *et al.*, 1990). Further, the formation of hydrogen peroxide or the formation of hydrogen ions as a product of primary (e.g., OH and O) and secondary species (e.g., H_2O_2, O_3, and ONOOH) as well as plasma-induced ionization of water molecules can lead to pH decreases. The influence of plasma on the pH of the treated liquid has been examined by Satoh et al. (2007). Water mixed with PBS buffer was exposed to a pulsed plasma system, which resulted in a decrease in the pH from values of around 7.3 to values between 3 and 4 by the dissolution of nitrogen oxides (NO_x) produced from air in the pulsed plasma. At the same time, increases in the liquids' conductivity were measured. In this study, differences in changes of the pH of the model solutions are most likely due to their different pk_a values and corresponding chemical reactivity. Catechol, which in contrast to the other model systems does not contain a carboxyl group, has a pk_a value of 9.45 (Schweigert *et al.*, 2000) and therefor is a more reactive compared to caffeic acid (pk_a = 4.62) and chlorogenic acid (pk_a = 3.33 – 3.96). With decreasing pk_a values of the hydroxycinnamic acid derivatives the impact of exposure to PPA on the pH of the solutions was less pronounced in general and especially during the first minute of treatment. Consequently and as expected, the most distinct effect on pH was measured in pure water having a pk_a value of 15.7. This is being supported by the calculated reaction constants k_1 and k_2 (Table 1), which provide evidence for the strong influence of the PPA treatment especially during the first stages of pH kinetics.

3.2 Plasma-induced accumulation of nitrate, nitrite and hydrogen peroxide in pure water
The significant accumulation of nitrate and nitrite in pure water upon exposure to PPA could be evidenced for all

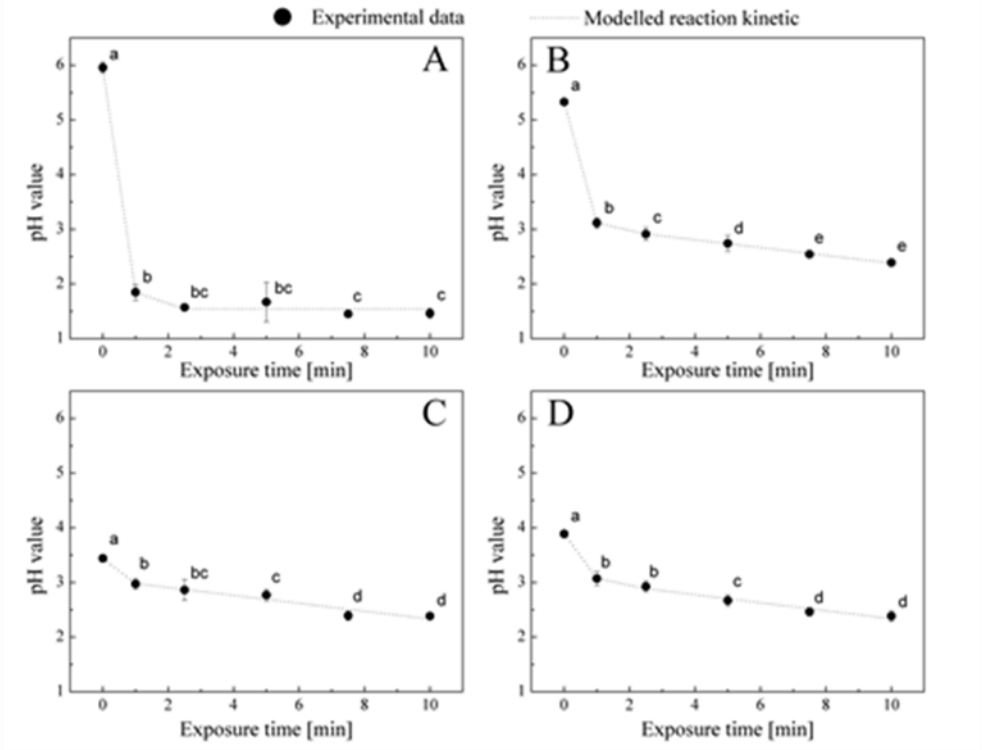

Figure 1: Change of pH in pure water (A), pyrocatechol (B), chlorogenic (C) and caffeic acid (D) solutions (10 mM) depending on plasma exposure time [min]. The results are presented as the mean of six replicates (n = 6) as experimental data. Different small letters (a-e) indicate significantly different values (p < 0.05, Tukey's test) for each sample. The dotted lines represent the reaction kinetic obtained from the applied biphasic model.

samples (Figure 2A-B) whereas the hydrogen peroxide concentration remained more or less stable at levels of about 4 to 6 mg L^{-1} (Figure 2C). Exposure to PPA for 1 min caused a steep increase in nitrate concentration from 10 to 1084 mg L^{-1} which tended to decrease to a final concentration of about 880 mg L^{-1} upon extending the exposure time to 10 min. Furthermore, the nitrite concentration rapidly rose from 0.1 to 25 mg L^{-1} within the first minute of treatment, then further increased to a maximum of 35 mg L^{-1} after 7.5 min and finally decreased to 12 mg L^{-1} extending the exposure to PPA to 10 min.

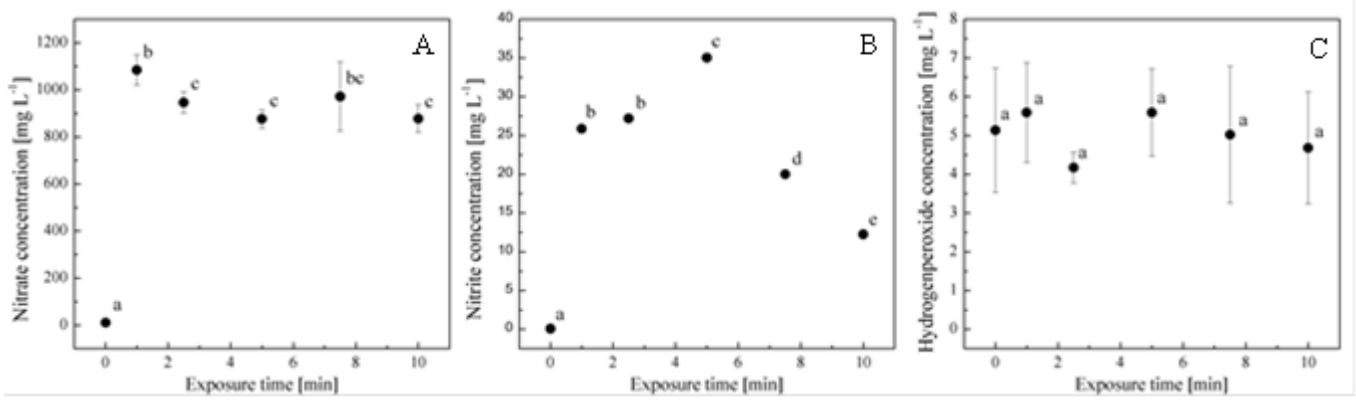

Figure 2: Concentrations of accumulated nitrate (left), nitrite (center) and hydrogenperoxide (right) in pure water depending on plasma exposure time [min]. The results are presented as the mean of six replicates (n = 6) as experimental data. Different small letters (a-e) indicate significantly different values (p < 0.05, Tukey's test) for each sample.

As "standard" air is assumed to be composed of nitrogen (78.08 %), oxygen (20.95 %), and argon (0.97 %) exposing it to a plasma source, in the general case, 28 species, including electrons, are considered in the plasma composition (Bacri & Raffanel, 1987). These species can be grouped into eight families: dinitrogen (N_2, N_2^+), atomic nitrogen (N, N^+, N_2^+, N_3^+), dioxygen (O_2, O_2^+. O_2^-), atomic oxygen (O, O^+, O_2^+, O_3^+, O^-), nitric oxide (NO, NO^+, NO^-), nitrogen dioxide (NO_2, NO_2^+, NO_2^-), nitrous oxide (N_2O, N_2O^+, N_2O^-) and argon (Ar, Ar^+, Ar_2^+, Ar_3^+). Schnabel et al. (2014) analyzed the composition of microwave PPA using identical experimental conditions. Via mass spectrometry they showed that 2.7 % of the working gas is converted into NO_2, NO, and a mixture of HNO_2, HNO_3, CO_2, and H_2O. As nitric oxide (NO) cannot coexist with ozone or atomic oxygen the formation of O_2, NO_2 and NO_3 proceed via oxidation reactions (Surowsky, Schlüter & Knorr, 2014). In the context of gas–liquid interfaces reactive nitrogen species (RNS) are further of special interest in this study as for instance through the reaction of NO with OH radicals or in the presence of O_2, the otherwise metastable nitric oxide reacts to nitrogen dioxide which is easily converted to N_2O_4, Nitrite (NO_2^-) and nitrate (NO_3^-). Once plasma comes into contact with water molecules, water dissociation reactions with electrons occur

(Melton, 1970) whereas the reaction rates greatly depend on the water content as well as on the electron energy and collision cross sections for water molecules with electrons (Locke, Lukes & Brisset, 2012). In this study, the

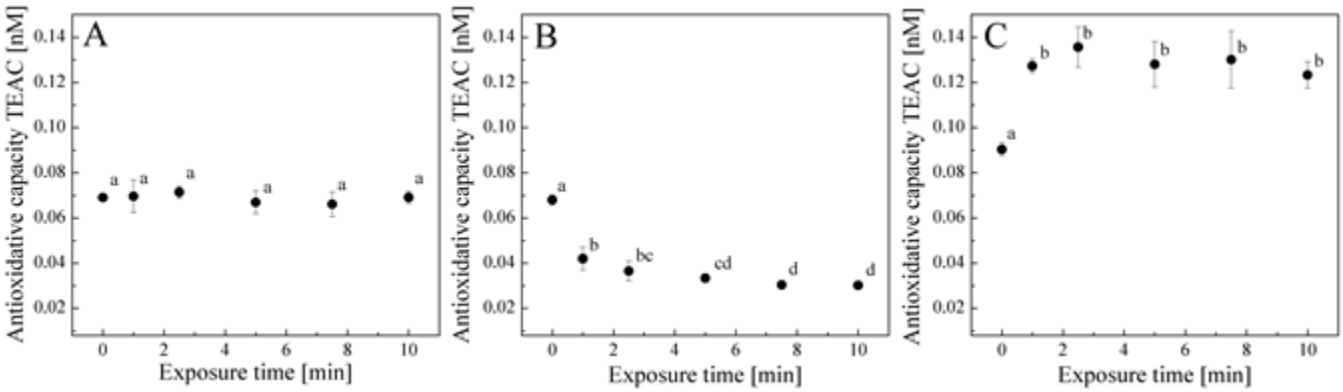

Figure 3: Change of the antioxidative capacity as measured by the TEAC assay in pyrocatechol (A, 1 mM), chlorogenic (B, 0.1 mM) and caffeic (C, 0.1 mM) adic solutions depending on plasma exposure time [min]. The results are presented as the mean of six replicates (n = 6). Different small letters (a-d) indicate significantly different values (p < 0.05, Tukey's test) for each sample.

accumulation of nitrate, nitrite and hydrogen peroxide caused by the exposure of pure water to PPA provides evidence for the presence of reactive species in the liquid phase, thus in principle fulfilling the theoretical premises for the formation of nitrophenolic compounds in phenolic model solutions.
of nitrophenolic compounds in phenolic model solutions.

3.3 Impact on antioxidative capacity of hydroxycinnimic acid solutions

While considering the impact of PPA treatment of the three model solutions used, three different effects were obtained: The antioxidative capacity of the pyrocatechol solution did not significantly change upon exposure to PPA for up to 10 min (Figure 4A), it decreased in chlorogenic acid solution (Figure 4B) and increased in caffeic acid solution ($p<0.05$, Figure 4C) whereas reaction kinetics of the latter two substances also follow the course of a biphasic reaction correlating with the data obtained regarding the plasma-induced pH decrease. In general plant phenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers. In this study, the major reactive species involved in the modification of antioxidant capacity of the model solutions are most likely associated with hydroxyl radicals and hydrogen peroxide (Sharma *et al.*, 1993). Applied to air, with certain humidity, the microwave plasma discharge induces the formation of hydroxyl radicals, hydrogen peroxide, and aqueous electrons, as well as several other species (Bednar, 1969; Spinks & Woods, 1976; Clements, Sato & Davis, 1987). The reaction mechanism may be similar to those in radiolytic processes such as γ-radiation, electron beam radiation, pulsed radiolysis and photochemical processes which produce radical and molecular species through excitation and ionization of the treated material, since the process involves excitation, ionization, and dissociation of water (Hughes, 1973). Further, hydroxyl radicals and hydrogen peroxide hydroxyl radicals can directly attack organic compounds and further lead to oxidation reactions and the formation of oxidation products with modified antioxidative characteristics. The oxidative coupling of phenols is well documented with o- and p-dihydroxyphenols (Cha, Berry & Lim, 1986; Ghosh & Misra, 1987; Cilliers & Singleton, 1990) and involves the formation of a reactive electrophilic quinone intermediate that can readily undergo attack by nucleophiles. It is described, that the presence of nucleophiles leads to the formation of addition products on different positions of the benzene ring, whereas the 2-position of the benzene ring in caffeic acid is the most electrophilic. Nucleophilic addition occurs preferentially here (Cheynier *et al.*, 1986). Thus the formation of oxidation products that involved mainly ether and carbon to carbon linkages involving the benzene ring is expected (Lundquist & Kristersson, 1986). Known reaction mechanisms concerning the autoxidation of phenols primarily involve the formation of quinones or semiquinone radicals as intermediates in presence of oxygen. Hereby, the initiation is postulated to occur by a mechanism favoring the spontaneous reaction of oxygen with abstractable hydrogen with the reaction being accelerated by participation of ROS. For caffeic acid, autoxidation of has been studied in some detail (Cilliers & Singleton, 1989; Cilliers & Singleton, 1990; Cilliers & Singleton, 1991) and involves the formation of a phenolate anion, which is supposed to be necessary to form a semiquinone via charge transfer with triplet oxygen. The formed semiquinone will then undergo further reaction. The triplet oxygen accepts the electron to become a superoxide anion radical in this process. It is clear that the reaction conditions such as time period, temperature, pH-value, oxygenation, concentration of the phenolic compounds as well as the number and position of the hydroxyl groups present, may greatly influence the extent of phenol oxidation (Kroll, Rawel & Rohn, 2003).

3.4 Plasma-induced changes in UV/Vis spectra of hydroxycinnimic acid solutions

Exposure to PPA induced changes in the exterior appearance of the samples. Except for pure water, the previously clear and colorless solutions of phenolic compounds had become brown-yellowish colored, partially sediment formation occurred. In general, obtained UV/Vis spectra indicated an increase of absorbance at low wavelengths around 200 nm. In case of caffeic and chlorogenic acid the two characteristic double peaks of the untreated substances at wavelengths of about 220/240 and 290/320 nm, respectively, rapidly decreased with increasing exposure time (data not shown). Those results provide preliminary indications on the plasma-induced degradation of the two model substances. Due to the optical characteristics of chlorogenic and caffeic acids and their resulting UV/Vis spectra the formation of nitrated adducts with absorption maxima at around 320 nm could not

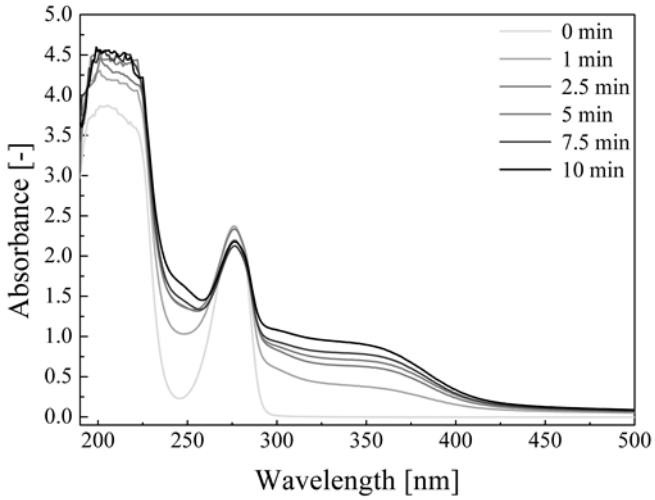

Figure 4: Plasma-induced changes in UV/Vis spectra in pyrocatechol solution (1 mM) dependent on plasma exposure time [min].

be proven. Cilliers and Singleton (1990) showed that a decrease of absorption at wavelength 326 nm indicates the involvement of the caffeic acid side chain in the occurring reaction. However, since not all this absorption is lost during exposure to PPA for up to 10 min, it has to be concluded that at least some of the side chain ethylene conjugation still exist in this crude mixture of oxidized products. The increase in absorption at 420 nm indicates an increase in visible brown color whereas the differences (and similarities) with the caffeic acid spectrum indicate the possibility of modified caffeic acid oligomers. Some side chain conjugation still exists in the oxidized products to give the 290- and 326-nm peak maxima, although the 326-nm absorbance relative to that at 290 nm is decreased to about half. An increase in absorbance can also be seen at 200 nm. In literature reaction products were shown to form dimer with a molecular mass of 358. With FAB in the positive ion mode, the dimethyl ester of caffeic acid $[M + H]^+$ peak at 387 was seen with fragments 355 $[M + H - CH30H]^+$, 323 $[M + H - 2CH30H]^+$, and 197 [M + H - caffeic acid methyl ester monomer]$^+$. Postulated mechanisms for the formation of such caffeicins were (i) phenolate ion formation, (ii) formation of semiquinone via reaction with oxygen and (iii) coupling of semiquinone to form the different structural isomers. It has been stated that enzymatic oxidation produces quinones much more rapidly at lower pH than autoxidation does (Cilliers & Singleton, 1990). The pH dependency indicates the involvement of the reactive phenolate ion in this reaction. The increase in absorbance at wavelengths around 200 nm was much less pronounced in plasma-treated pyrocatechol solutions (Figure 4). Under physiological conditions catechol is not auto-oxidized (Irons & Sawahata, 1985), except in the presence of heavy metals (Schweigert *et al.*, 2000).

Because catechols readily undergo oxidation to form semiquinone radicals and quinones, which are in some aspects more reactive than the catechols, the deleterious effects of these species has also been considered. Spectral analysis indicated i) a significant decrease of absorbance at 276 nm and ii) a slight increase of absorbance at 319/355 nm with increasing exposure to PPA, thus indicating the formation of highly chromogenic p-nitrophenol (Daneshvar, Behnajady & Zorriyeh Asghar, 2007). Based on those results and corresponding to data in literature showing pH dependent absorption maxima of 2- and 4-nitrophenol at 275 and 319 nm (Egerton *et al.*, 2005), respectively, RP-HPLC chromatograms were recorded at wavelength of 274 and 319 nm which were found to be the absorption maxima of the plasma-treated sample solutions.

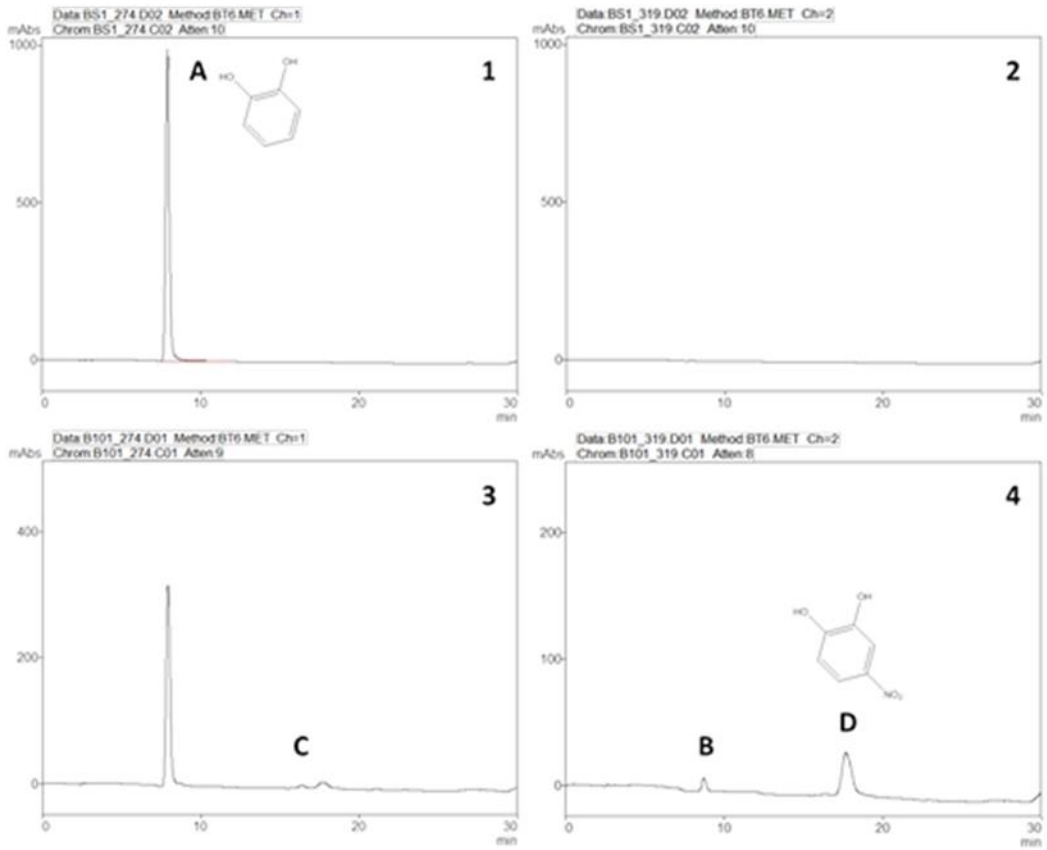

Figure 5: Exemplary RP-HPLC chromatogram of the separation of pyrocatechol detected at 274 (1) and 319 nm (2) and plasma-induced adducts RPA, RPB and RPC detected at 247 (3) and 319 nm (4) in pyrocatechol solution (10 mM) after a 10 min exposure to PPA. Code: A = Catechol; B = RP A; C = RP B; D = RP C.

3.5 Formation and identification of nitrophenolic compounds

The sample solutions exposed to PPA were characterized by a rather complex composition indicated by numerous peaks that became apparent in recorded RP-HPLC chromatograms (Figure 5). Therefore and with regard to the intention of this study – to provide evidence for the formation of nitrophenolic compounds by PPA treatment - the analyses focused on several prominent features. Following exposure to PPA three new main peaks could be detected for the pyrocatechol samples accompanied by a degradation of the initial pyrocatechol concentration (Figure 5A). The loss of pyrocatechol due to plasma treatment was quantified using pyrocatechol as standard. Corresponding to the loss in pH the degradation kinetic of pyrocatechol was found to have a biphasic character.

Table 2 *Means (± sd; n = 6) of reaction rate constants k_1 and k_2 for a biphasic fit of the plasma-induced degradation of pyrocatechol and the formation of RP A and RP C as welll as reaction rate constants k_1, k_2 and k_3 for a triphasic fit of formation and degradation of RP B. Goodness-of-fit is presented via R^2 and RSME values.*

	k_1 [-]	k_2 [-]	k_3 [-]	R^2	RSME
Degradation Pyrocatechol	-3.36 (± 0.61)	-0.79 (± 0.06)	-	0.9995	0.0739
Formation RP A	3.59 (± 0.28)	0.1 (± 0.03)	-	0.9980	0.1382
Formation RP B	5.78 (± 0.31)	-6.0 (± 0.28)	-0.04 (± 0.01)	0.9748	0.2647
Formation RP C	1.51 (± 0.10)	0. 22 (± 0.11)	-	0.9953	0.0634

During the first minute of exposure to PPA, the pyrocatechol content was reduced to 71.1 % followed by a nearly linear decrease to 39.4 % upon treatment for up to 10 min (Figure 6A). In general, the AUC of the plasma-induced pyrocatechol reaction products increased with treatment time (Figure 6B-D). The AUC of reaction product A (RPA) with a retention time of 8.4 min increased with increasing exposure to PPA whereas it was not detectable in the untreated samples. Besides the presence of unmodified pyrocatechol (m/z = 109), with a retention time of 7.9 min, the LC/MS analysis revealed a prominent m/z values of 169 for RPA which is most likely attributed to the formation of reaction products during RP-HPLC analysis, for instance caused by the interaction of pyrocatechol and acetic acid present in the eluent. A second product (RP B) with a retention time of 16.5 min and a prominent m/z value of 217 was detected as well as a third reaction product (RP C) with a retention time of 17.5 min and prominent m/z values of 154 and 309 (Figure 7). As already discussed for the pH decrease the reaction kinetics regarding the formation of RP A and RP C could also be described as biphasic. In both cases the first phase of treatment is characterized by a strong increase in determined AUC for the respective reaction product. The reaction constant k1 of the formation of RP A was in the range of that for the degradation of pyrocatechol whereas k1 for the formation of RP C was about half of that (Table 2). In case of RP B, a triphasic reaction kinetic was detected characterized by a steep linear increase of RP B during the first phase and ensuing biphasic degradation in the second and third phase.

As proposed regarding the reaction mechanism in caffeic acid solution, the obtained triphasic kinetic may be attributed to the formation of phenolate ion, subsequent oxidation to form quinones, and nitration. As expected the only prominent m/z value of the untreated chlorogenic acid sample was 353 whereas m/z value of 179 was detected in untreated caffeic acid sample. Regarding the latter substance these m/z values were barely detectable after exposure to PPA for 10 min, instead the m/z value of 224 was prominent. In case of plasma treated chlorogenic acid a m/z value of 398 was found via LC/MS analysis. Proposed chemical structures and formula of possibly formed mono-nitrated phenolic compounds are depicted in Figures 7 and 8.

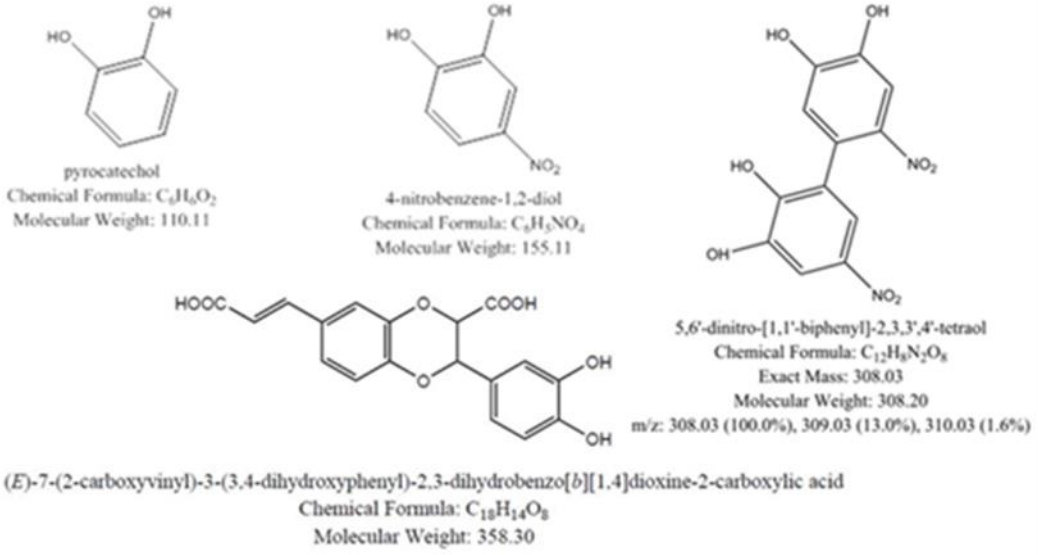

Figure 6: Plasma-induced degradation of pyrocatechol (A) and plasma-induced formation and degradation of RPA (B), RPB (C) and RPC (D) depending on plasma exposure time [min]. The results are presented as the mean of fifteen replicates (n = 15). Different small letters (a-f) indicate significantly different values ($p < 0.05$, Tukey's test) for each sample.

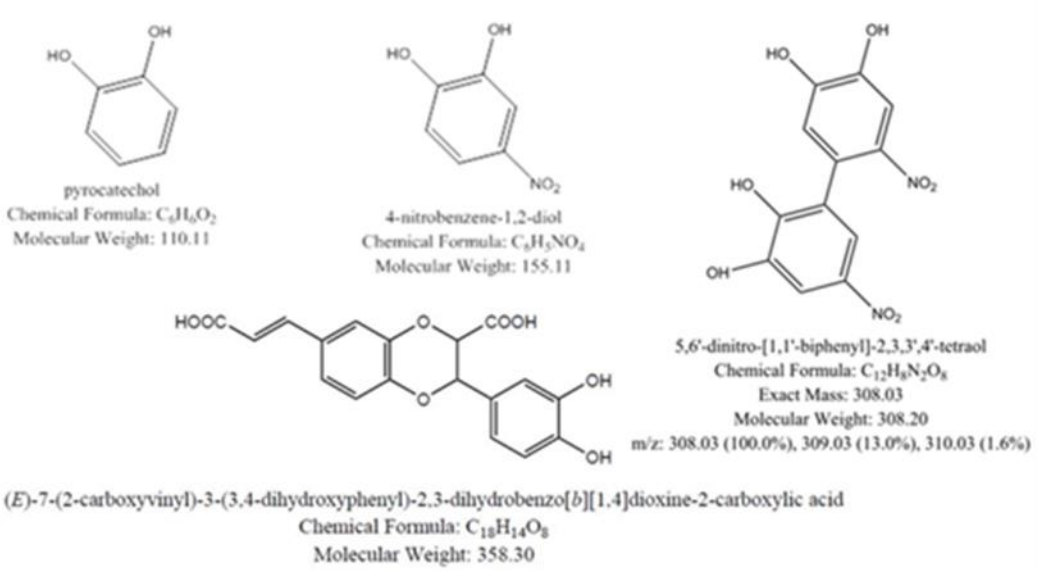

Figure 7: Proposed products of pyrocatechol under conditions of the PPA-mediated reaction

In case of pyrocatechol the formation of 4-nitrobenzene-1,2-diol (molecular weight 309) and resulting dimers seems highly probable (Figure 7). Dimerization and polymerization reactions are also supported by the formation of sediments in the pyrocatechol solution. Due to their insolubility, the analysis of these polymers via LC/MS was not further explored in this study and ought to be the subject of further investigations. With regard to chlorogenic acid the plasmainduced formation of (1*S*,3*R*,4*R*,5*R*)-3-(((*E*)-3-(4,5-dihydroxy-2-nitrophenyl)acroyl)oxy)-1,4,5-trihydroxycyclo-hexane- 1carboxylic acid (molecular weight 399.31) provides one possible explanation for the detected m/z value of 358 (Figure 7) whereas the formation of (E)-3 (4,5-dihydroxy-2-nitrophenyl)acrylic acid (molecular weight 225.16) is assumed to be formed during exposure of caffeic acid solution to PPA (Figure 8).

Underlying plasma-induced reaction mechanisms of oxidation, nitration and even nitrosation are manifold. Often the first step in the oxidation is attributed to the reaction of hydroxyl radical. According to the conditions, as for instance the oxidability of radicals formed, the second step may be dimerization, disproportionation or oxidation by oxygen and oxygen radicals. This study mainly aimed at providing evidence for the nitration of phenolic compounds by exposure to PPA. Nitration and nitrosation reactions result from electrophilic reactions unfavoured by electron withdrawing substituents. In literature, it is described, that the formation of nitro- and nitrosophenols cannot be attributed to the reaction of NO or NO_2 with phenoxyl radical resulting from the oxidation of phenolic compounds by hydroxyl radicals. Further it was shown, that nitrous acid is involved only in acidic solutions and does not induce the nitration of phenolic compounds (Matykiewiczová *et al.*, 2007) whereas nitration and nitrosation can be related to the presence of nitrogen dioxide in the solution. The plasma-induced enhancing effect on nitration and cannot be explained only by the formation of nitrous acid. It seems likely that for instance the protonation of N_2O_4 favors its electrophilic reaction with the phenolic ring.

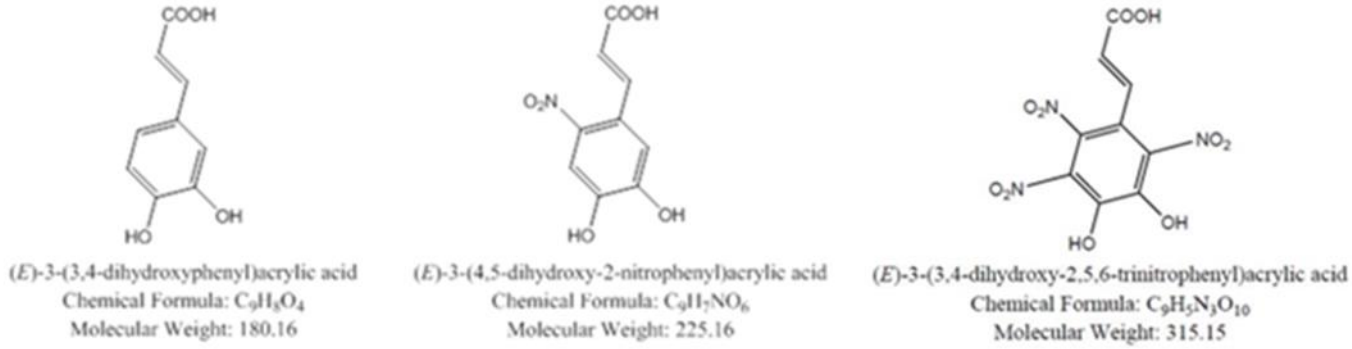

Figure 8: Proposed products of chlorogenic acid under conditions of the PPA-mediated reaction.

Further, the reaction of OH radicals with organic compounds can lead to hydrogen abstraction, hydroxyl electrophilic addition, and direct electron transfer. Typical targets for hydrogen abstraction are saturated aliphatic hydrocarbons and alcohols, whereas compounds such as phenols can be electrophillically attacked by the addition of OH radicals to the C=C bonds. The organic radical R typically forms ROO radicals, which subsequently react to compounds such as hydroperoxyl radicals and hydrogen peroxide as well as to aldehydes and acids (Lukes, Locke &

Brisset, 2012). Hydrogen peroxide (H_2O_2), as another possible reaction partner, is compared to OH and O_3, a very long-living ROS, which is highly water-soluble, able to oxidize as well as to reduce organic compounds and which increases the overall oxidizing power of a plasma. For the formation of H_2O_2, the solution pH and conductivity play an important role as lower liquid-phase pH values tend to lead to an increased production of hydrogen peroxide (Thagard, Takashima & Mizuno, 2009). H_2O_2 is involved in various important reactions such as the formation of OH, peroxynitrite, and HO_2. In the presence of an organic compound such as phenol, the hydroxyl radicals react with the organic compound to produce oxidation products. Further it can be assumed that hydroxyl radicals formed from microwave driven plasma torch react with phenol to produce oxidation products which again react with hydroxyl radicals. Regarding the reaction mechanism for the oxidation of phenol via hydroxyl radical attack it has been reported that the primary oxidation products of phenol due to hydroxyl radical attack are hydroquinone (1,4-dihydroxybenzene), catechol (1,2-dihydroxybenzene), and resorcinol (1,3-dihydroxybenzene). The secondary products are pyrogallol (1,2,3-trihydroxybenzene), 1,2,4-trihydroxybenzene, pquinone (l,4-benzoquinone), 2-hydroxy-1,4-benzoquinone (Gurol & Singer, 1983; Gurol & Singer, 1983; Okamoto *et al*., 1985). Furthermore, the generation of highly reactive peroxynitrites acting as peroxidizing agent is very likely. The action of peroxynitrite greatly depends on the pH of the liquid (Bian *et al*., 2010; Bermúdez-Aguirre *et al*., 2013), direct oxidation reactions may occur, whereas peroxynitrite decays into OH and NO_2 under acidic conditions, which subsequently initiate indirect reactions (Surowsky, Schlüter & Knorr, 2015).

4. Conclusion

A basic hypothesis of this study – the plasma-induced formation of nitrated phenolic compounds- could be clearly established and is supported by the observed m/z value of the mono-nitrated reaction products in negative ionization mode for all three focal model substances. Also, the identified isotope pattern fitted to the natural isotopic abundance of carbon, i.e. the number of carbon atoms remained unchanged in the molecules of the mono-nitrated reaction products. The results show that the nitration reactions, the formation and degradation of reactants are dependent on the exposure time to PPA. The main reactions observed when the liquid model phenolics (pyrocatechol) as well as characterized derivatives of hydroxycinnimic acid (chlorogenic acid and caffeic acid) were exposed to PPA were attributed to oxidation, nitration and polymerization of the phenolic compounds. However, further experiments and analyses are needed to clarify their chemical composition. Underlying reaction mechanism could not be fully clarified and need to be investigated in ongoing research in order to use PPA as an innovative approach for the production of aromatic nitrophenolics for instance by the aeration of phenolic solutions with PPA without addition of chemicals.

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4. Potential applications along value-added chains of food

With the constant development of plasma devices suitable for the tailor-made treatment of a wide range of different raw materials, intermediates and products in the food sector, the applicability of the CAPP technology extends across entire value chains and consequently offers manifold approaches, which is also reflected in numerous publications in literature [21, 31, 32, 36, 100-107]. These approaches mainly include the

i. control of biofilms and treatment of processing surfaces, waste water and exhaust, the
ii. treatment of seeds and sprouts, as well as of
iii. fresh produce,
iv. dry produce, and
v. end products, and further
vi. in-package treatment, and the
vii. modification and sterilization of food packaging polymers,

which are summarized and described below and in figure 15.

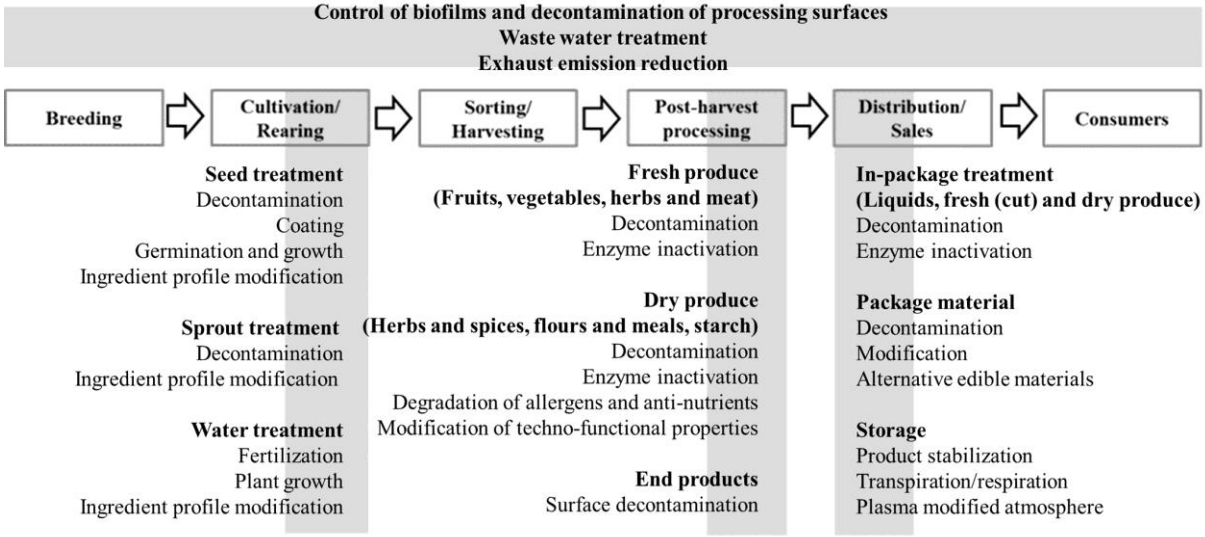

Figure 15: Potential applications of the CAPP technology along value-added chains of food.

Control of biofilms and treatment of processing surfaces, waste water and exhaust

There are applications of CAPP in the food sector, which can be assigned at any stage of value chains and at various stages of processing lines, as for instance the control of biofilms, the decontamination of processing surfaces, as well as exhaust and waste water treatment. Pei *et al.* [36] reported effective 25.5 µm-thick *Enterococcus faecalis* biofilm inactivation using a handheld, mobile plasma jet powered by a 12 V DC battery operated in open air without any external gas supply. A study conducted by Niemira *et al.* [21] showed the reduction of mature

Salmonella biofilms by up to 2.13 log CFU/mL following 15 s treatment with cold plasma. As a number of pathogens can form chemical-resistant biofilms making them difficult to remove from food contact surfaces, CAPP, as a contact-free waterless method, may therefore have a practical application for the sanitation of conveyor belts, equipment, and other food contact surfaces, where a rapid, dry antimicrobial process using no chemical sanitizers is required. Rowan *et al.* [100] developed a pulsed plasma gas-discharge (PPGD) system for the plasma-assisted decontamination of chilled poultry wash water and showed that treatment in the plasma generation chamber for up to 24 s at 4 °C reduced *Escherichia coli* NCTC 9001, *Campylobacter jejuni* ATCC 33560, *Campylobacter coli* ATCC 33559, *Listeria monocytogenes* NCTC 9863, *Salmonella enterica* serovar Enteritidis ATCC 4931, and *S. enterica* serovar Typhimurium ATCC 14028 populations to nondetectable levels (≤8 log CFU/ml). The results indicate that the CAPP technology offers an exciting complementary or alternative approach for treating raw poultry wash water and for preventing cross-contamination in processing environments.

Seeds and sprouts

In recent years, quite a number of publications dealt with the plasma treatment of foodstuffs, as the nontoxic and low-cost CAPP technology is applicable in dry and wet environments. It further allows a treatment of fresh food and virtually every conceivable surface in batch or inline processes along the whole value chain of food production, and could be implemented as an alternative to ozone or chlorine dioxide treatment. At the outset of the value-added chain of plant-based produce, the CAPP application on plant seeds and sprouts for decontamination, coating, acceleration of germination and growth, as well as the modification of ingredient profiles has attracted broad interest in agricultural and food sciences. Mihai *et al.* [101] investigated the effect of CAPP on radish seeds and showed that plasma treatment had little effect on the germination rate, but influenced the early growth of seeds. Compared to the control seeds, sprouts, and roots of plasma treated seeds were longer and heavier. Sera *et al.* [102] showed and discussed the influence of cold plasma treatment on germination enhancement of wheat and oat caryopses in wider context stimulating wheat and oat corns by cold plasma discharge (500 W, air gas flow of 200 ml/min, up to 2400 s). CAPP treatment caused an eroded surface on the wheat seed coat and inhibited the germinating acceleration of wheat in first days, but enhanced rootstalk was observed on plants grown from seeds treated for medium time. Germination of oat seeds was not affected, but rootlet generation at plants grown from treated seeds was accelerated. Different content of phenolic compounds between control sprouts and sprouts from treated seedlings illustrated changes in metabolism processes in both tested species. A recent study by Shiratani *et al.* [103] investigated plant growth response of seeds on their growth to CAPP treatment for 5 plant species; Radish sprout (*Raphanus sativus L.),* rice (*Oryza Sativa*), Zinnia, *Arabidopsis L. Thaliana* and *Plumeria* and found an increase in average length of Radish sprout, rice, *Arabidopsis Thaliana*, *Plumeria* and *Zinnia*, by 250 %, 80 %, 60 %, 30 %, and 20 %, respectively. They further obtained correlation between the growth enhancement and O_3 and NO_x concentration and concluded that the optimum radical dose for the growth enhancement depends on plant species. Apart from these application possibilities, the CAPP technology was also tested for decontamination

and coating of seeds, as well as for producing plasma treated water with modified properties and chemical composition, which in turn may affect plant growth process and subsequently agriculture produce quality. Selcuk *et al.* [104] determined the efficacy of a self-designed low pressure cold plasma system using air gases for the inactivation and/or elimination of two pathogenic fungi, *Aspergillus* spp. and *Penicillum* spp. artificially contaminated on seed surface. The study showed that plasma treatment reduced the fungal attachment to seeds below 1 % of initial load depending on the initial contamination level, while preserving germination quality of the seed. A study to determine, if an alternate seed treatment approach based on plasma chemistry would offer a more viable alternative over traditional seed coating technologies, was conducted by Volin *et al.* [108]. The germination characteristics of five agricultural species were modified by coating the seed surfaces with macromolecules from a CAPP process using a rotating plasma reactor. The type of coating was determined by the source gas (carbon tetrafluoride or octadecafluorodecalin), and coatings were typically much less than 5.0 μm in thickness. The use of the two different hydrophobic source gases resulted in a significant delay in germination compared with untreated control seeds of radish (*Raphanus sativus*) and two pea cultivars (*Pisum sativum* 'Little Marvel', *P. sativum* 'Alaska'), whereas the degree of delay was dependent on the amount of coating applied. An increased thickness of coating resulted in a greater delay in germination. In turn, seeds treated with cyclohexane resulted in a significant acceleration in germination percentage for soybean. The major mode of action of the plasma coatings was largely on the rate of imbibition, as determined by tests of water uptake. These results demonstrate that CAPP offers a potentially important technique to modify seed germination characteristics in agricultural plant species. Park *et al.* [109] reported on effects of plasma-treated water on plant development. Water exposed to non-equilibrium plasma discharges is characterized by plasma-induced change of its properties and chemical composition, which in turn may affect plant growth process, as water composition plays a key role in plant germination, development, and growth. The study found a significant drop in pH following plasma treatment accompanied by an increase in nitrate, nitrite, and hydrogen peroxide concentration in the treated water. The results concerning the effects of plasma treated water on plants, which differed upon different plants, were summarized as promising.

Fresh produce

Further up the value-added chain of agricultural produce for the food sector, the applicability of the CAPP technology was tested for a variety of fresh plant and animal-based products, as fruits and vegetables, and meat. It was shown that the post-harvest chain of fresh agricultural produce offers ample opportunities for rendering of innovative applications of the CAPP technology. As formerly mentioned, the treatment of whole pieces and voluminous types of fresh produce requires feasible plasma setups and respective application techniques as PPA and PPW. Schnebel *et al.* [105] contaminated five different fresh produces with seven different microorganisms, *e.g.* bacteria, yeasts and endospores and subjected them to a treatment with PPA leading to reduction factors of microbial load greater than 6 log. Sensory examinations showed only little influences in texture, appearance and odor. When introducing PPA into distilled water or tap water, the generated PPW may be applied for the

decontamination of packaging material and fresh produce. In another study, Schnabel *et al.* [30] tested the applicability of PPW as a new and innovative method for the generation of antimicrobial active water. They contaminated PET stripes, fresh-cut lettuce, and fresh sprouts with six different bacteria; *Escherichia coli* K12 (DSM 11250), *Pseudomonas fluorescens* (DSM 50090), *Pseudomonas fluorescens* (RIPAC), *Pseudomonas marginalis* (DSM 13124), *Pectobacterium carotovorum* (DSM 30168) and *Listeria innocua* (DSM 20649), which were treated with PPW. Following treatment for up to 5 min, decreases in bacterial loads of up to 6 log were detected for *P. fluorescens* (DSM-strain) on PET, and *P. marginalis* and *P. carotovorum* on salad. The inactivation efficacy was found to be lower for all other bacteria and specimen, which further were only marginally affected in their texture and the appearance after 8 days of storage. But the CAPP technology is also applicable to fresh produce of animal origin. Fröhling *et al.* [106] determined the impact of PPA treatment on quality (color, pH, fluorescence, and reflectance) and safety (aerobic viable count) of porcine *musculus longissimus dorsi* (MLD). It was shown that, following exposure to PPA, the aerobic viable count of MLD remained between 10^2 and 10^3 CFU/g during the storage period of 20 days at 5 °C. In comparison to untreated meat samples, color measurements revealed increased a-values and decreased b-values of pork meat caused by PPA treatment. The authors concluded that improved plasma process design is required in order to obtain significant microbial reduction without affecting the product quality and to guarantee consumer's acceptance.

Dry produce

Besides its applicability to fresh produce of plant and animal origin, the CAPP technology further offers the potential for post-harvest treatment of dry produce including a wide variety of possible objectives. Also in this field extensive research has been conducted during the past years. Kuloba *et al.* [110] used low-temperature nitrogen plasma to study its effect on the polyphenol content in green tea leaves. Green tea leaves were withered using a DBD chamber under varying the exposure time. They found the highest polyphenol content of 78.56 mg g^{-1} in tea, which was subjected to CAPP for 1 h. Another process goal in the area of CAPP treatment of dry produce is the effective decontamination of herbs and spices. Hertwig *et al.* [31] investigated the decontamination efficiency of PPA for three different types of herbs and spices (pepper seeds, crushed oregano and paprika powder) with various surface-to-volume ratios. These were subjected to PPA for up to 90 min. The native microbial flora of the pepper seeds and the paprika powder was reduced by more than 3 $\log_{10}$ following 60 min PPA treatment. However, treatment of red paprika powder resulted in a considerable loss of redness following exposure to PPA for ≥5 min, whereas the treatment had only a minor impact on the color of pepper seeds and oregano. Apart from the approach to inactivate undesirable and pathogenic microorganisms on surfaces of dry food produce, some researchers started to investigate the effects of CAPP technology on techno-functional properties of dry intermediates and products using the surface modifying and functionalizing properties of plasma. Chen [111] investigated the impact of low-pressure plasma treatment in air on the properties of long-grain brown rice. The authors determined the microstructure of the brown rice surface, and the cooking, textural, and pasting properties, and found that

exposure to plasma resulted in etching of the brown rice surface. Allowing water to be easily absorbed by the rice kernel during soaking led to a reduction in cooking time, elongation ratio, width expansion ratio, water absorption, and cooking loss of brown rice. Plasma further modified the pasting properties of the rice, as the starch structure of brown rice was influenced by low-pressure plasma. Similar plasma-induced effects were reported for basmati rice [112]. Lii *et al.* [113] exposed granular starches of nine botanical origins to low pressure glow plasma generated in air. Starches were partly oxidized to carboxylic starches, and partly depolymerized when their affinity to plasma depended on their botanical origin. The authors claimed that treatment with glow plasma may offer an alternative method of waste-less dextrinization of starches. Misra *et al.* [114] explored the possible effects of CAPP as a means to change the structural and functional properties of strong and weak wheat flours. Considering that previous studies have demonstrated that ozone modifies the functional properties of wheat flour [115], they generated plasma high in ozone concentration using a DBD device and air as the working gas. Plasma treatments were found to result in a voltage and treatment time-dependent increase in the viscoelasticity of the dough produced from the wheat flour. Those effects were attributed to the alteration of the secondary structure of gluten proteins, following measurements via FTIR spectroscopy. The interaction between CAPP and whey protein isolate model solutions was investigated by Segat *et al.* [116]. They found an increase in yellow color and a minor reduction in pH value, which they attributed to reactions of reactive oxygen and nitrogen plasma species with WPI. Mild oxidation in the proteins occurred upon exposure to plasma for 15 min accompanied by an increase in carbonyl groups and surface hydrophobicity and besides the reduction of free SH groups. Those results point to the effects on amino acid residues. Moreover, the authors correlated the reduction of free SH groups to the aggregation among proteins or a strong oxidative effect on cysteine. Dynamic light scattering revealed a certain degree of unfolding, as confirmed by high performance liquid chromatography profiles. Those plasma-induced protein structure modifications may be responsible for the improvement in foaming and emulsifying capacity the authors determined.

End products

The CAPP technology may further be used as a kind of final treatment of processed food end products with the goal of surface decontamination or modification. In literature, some examples are given. Song *et al.* [65] evaluated the efficacy of CAPP in decontaminating sliced cheese and ham inoculated by 3-strain cocktail of *Listeria monocytogenes* (ATCC 19114, 19115, and 19111, LMC) and found that the microbial log-reduction increased with increases of input power and plasma exposure time. Results obtained indicated that the inactivation effects of CAPP on *L. monocytogenes* are strongly dependent on the type of food. As formerly mentioned, Misra *et al.* [84] demonstrated the capability of DBD plasma to enhance the surface hydrophobicity of freshly baked biscuits, thus evidencing possible plasma applications in the industrial preparation of biscuit and cracker, where post-baking oil spray is desired.

In-package treatment

New trends aim to develop in-package decontamination, which offers nonthermal treatment of foods post-packaging and further minimizes the possibility of post-process recontamination. Rød *et al*. [117] investigated the application of CAPP for decontaminating sliced ready-to-eat meat product (bresaola) inoculated with *Listeria innocua*. CAPP treatment of the inoculated samples were conducted inside sealed linear-low-density-polyethylene bags containing 30 % oxygen and 70 % argon and resulted in a reduction of *L. innocua* ranging from 0.8 ± 0.4 to 1.6 ± 0.5 log cfu/g. Whereas no significant effects of plasma exposure time and intensity was found, multiple treatments with a 10 min interval further increased reduction of *L. innocua*. Surface color changes included loss of redness during storage regardless of plasma treatment. A study performed by Misra *et al.* [118] investigates the effects of CAPP generated within a sealed package from a DBD on the physical quality parameters and respiration rates of cherry tomatoes. Observed differences among weight loss, pH and firmness for control and treated cherry tomatoes were insignificant towards the end of storage life, whereas changes in respiration rates and color of tomatoes were not drastic. The results implicate that in-package CAPP treatment could be employed as a means for decontamination of cherry tomatoes while retaining product quality. A different study conducted by Misra *et al.* [28] studied the capability of in-package CAPP treatment of strawberries generating CAPP inside a sealed package containing strawberries, using a DBD setup in the filamentary regime. A drastic change in respiration rate of strawberries did not occur, although the modified gas composition induced through complex plasma chemistry may persist for several hours inside the package. Thus, results demonstrated the ability of in-package CAPP treatment to reduce the background microflora present on strawberries without inducing significant physiological (respiratory) stress or adversely affecting the color and firmness.

Modification and sterilization of food packaging polymers

While the CAPP technology was originally developed to increase the surface energy of polymers, enhancing adhesion and printability, it has recently emerged as a powerful tool for surface decontamination of food packaging materials. Due to the environmental issues associated with the packaging waste, the development of biodegradable packaging alternatives is required, too. Pankaj *et al.* [119] subjected sodium caseinate films to DBD plasma. The films were physicochemically characterized for the plasma-induced effects as function of applied voltage and treatment times. Surface roughness and surface hydrophilicity of plasma treated films was increased, whereas glass transition temperature of all the CAPP-treated films was found to be lower compared to the control film. These effects were accompanied by an increase in the O/C atomic ratio showing the formation of new oxygen-containing groups on the film surface. Further, a disruption in the inter-helical structure without any change in the helical configuration of the protein molecules was suggested via XRD and FT-IR spectroscopy. Pankaj *et al.* [120] studied the effects of DBD plasma on the surface topography, thermal behavior, chemical composition and water vapor permeability of high amylose corn starch films. Again, the plasma treatment significantly increased the surface roughness and hydrophilicity of the starch films, whereas XRD and FTIR

spectroscopy confirmed the increase in surface oxygen content and appearance of new O=C–O groups on the film surface. Using the CAPP technology to modify biodegradable polymers consequently shows the compatibility of two environmentally-friendly strategies in food technology: (i) CAPP treatment for the decontamination of food packaged with (ii) biodegradable materials.

Given its diversity of application possibilities, the plasma technology is applicable to moist and dry surfaces from animal and vegetable origin though the complexity of plasma chemistry makes the explicit elucidation of the underlying reaction pathways a challenging and up to date not fully resolved task.

4.1 Selected examples for technology integration: processing of legumes

The steadily increasing world population has motivated the development of new strategies for the coverage of the required protein consumption in recent years. Emphasis is currently being placed on sustainability, low costs, and nutritional properties of plant-based proteins as an alternative to the established animal-based proteins that are currently in the market. Within the context of the protein crop strategy, governments are searching for alternative, sustainable local products for being integrated in long-term protein utilization strategies. As a consequence, a revival of domestic grain legumes, as peas, beans and lentils, has been encouraged in Germany and other European countries, as legumes constitute a promising alternative to the critically considered use of soy. Providing a protein content of approximately 40 %, soybean represents a valuable protein source for food and feed [121-123], further the process chains for soybean and soybean proteins are well-developed. But also domestic grain peas (*Pisum sativum*) are rich in starch, fiber, vitamins, and minerals; and with protein contents ranging from 23.3 % to 31.7 % among different pea varieties [124, 125], they have received more attention as alternative sources for providing protein-rich intermediates [126, 127]. Compared to soybeans, pea proteins provide higher levels of lysine, but less sulphur containing amino acids [128, 129]. Pea flour does not contain any gluten and consequently could be a useful contribution for the production of gluten-free foods [130, 131]. Moreover, peas and innovative products developed thereof could contribute to secure sufficient, sustainable foods for vegetarians and vegans. Because of bringing high yields, providing high protein content and ripening comparatively early under the local climatic conditions, the variety ‘Salamanca’ is widely cultivated in Germany. Comparing different cultivars of *P. sativum* ‘Salamanca’ was further found to have the most desired flavonoid glycoside profile referring to high concentrations of quercetin glycosides and exemplarily the acylation with p-coumaric acid.

As the CAPP technology was found to specifically react with plant secondary metabolites and further modifies plant surfaces, innovative plasma applications can be found in food technology related to high-protein plant materials. Two of them, namely the plasma-induced stress response of plants’ secondary metabolism, as well as the targeted modification of flour

and protein functionalities, may play a substantial role in current research dealing with the use of alternative plant protein sources, as protein functionality plays a key role in the

i. improvement of existing products, the
ii. development of new products, and the
iii. utilization of protein waste products as new ingredients.

In order to highlight the advantage of the CAPP technology of being applicable at various stages of processes and value chains, CAPP experiments investigating the above mentioned approaches were conducted along the value-added chain of legumes working with the example of peas (Fig. 16).

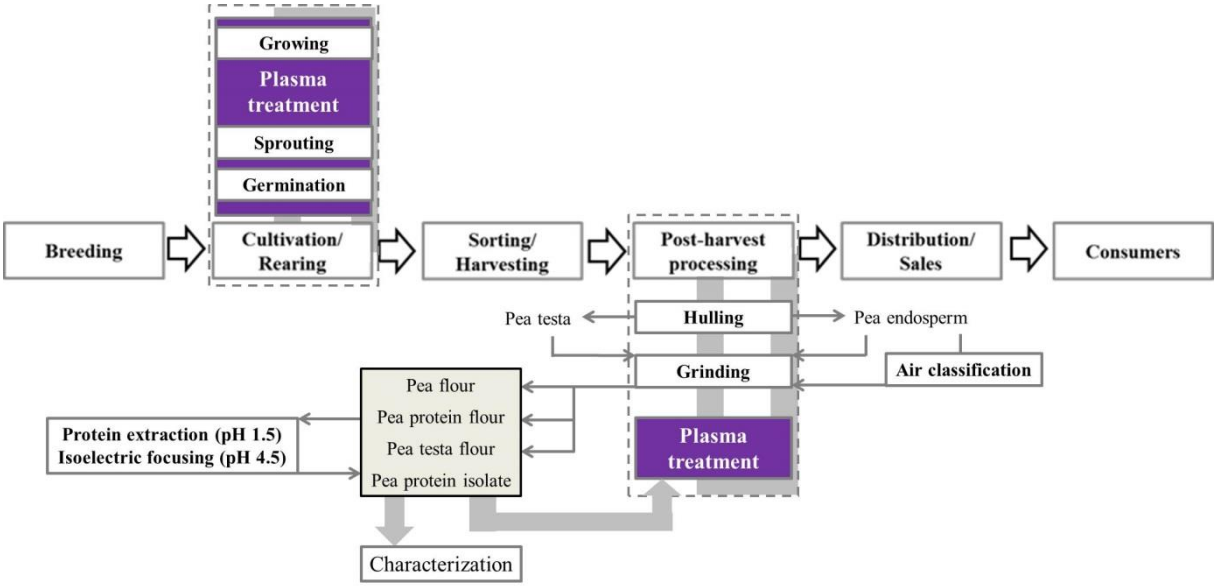

Figure 16: Potential integration of the CAPP technology into the value-added chain of peas.

At the outset of the value-added chain of plant-based produce, the CAPP application on plant seeds and sprouts for decontamination, coating, acceleration of germination and growth, as well as the modification of ingredient profiles has attracted broad interest in agricultural and food sciences. Bormashenko et al. [132] reported the possibility to modify the surface wetting properties of a diversity of seeds, including lentils, beans and wheat, by cold radiofrequency air plasma treatment. Air plasma treatment led to a dramatic decrease in the apparent contact angle, in turn changing the wettability of beans and lentils and giving rise to a change in the water absorption (imbibition) of the seeds. Further, plasma processing of plant material for food may change the structure of the food matrix, which is of great importance for the bioavailability of phytochemicals ingested in the matrix.

In particular, germinated legumes are excellent sources of non-acylated and monoacylated triglycosides of quercetin and kaempferol [133, 134]. In the postharvest chain of legumes, germination is the key step for improving their nutritional quality and functionality [135-137]. In order to effectively increase the biosynthesis of secondary metabolites, biotic and abiotic stresses, as well as chemical and physical elicitors were used in numerous studies [138, 139]. Flavonoids are significantly involved in the stress responses of plants [140-142] and have, due

to their potential cardioprotective and anti-carcinogenic effects, gained considerable interest in recent years [143-145]. In plants, flavonoids partially act as shielding components against excessive radiation both, in the photosynthetically active, and in the UV wavelength ranges, and further play an important role in the plants' defense systems, e.g. against ROS as effective antioxidants [146]. CAPP offers a source of ROS; e.g. atomic oxygen (O), ozone (O_3), hydroxyl radical (OH•) and RNS; e.g., N_2, NO, NO_2, nitric oxide radical (NO•) as well as UV-A and UV-B radiation [52] and thus, may be used as a targeted tool for the stress-induced modification and intensification of plants' secondary metabolism. Hence, postharvest application of CAPP on plant materials may offer the possibility of eliciting flavonoid synthesis without damaging the plant tissue [147]. Sookwong et al. [148] evidenced that plasma processing could enhance and promote germination of rice seeds by improving physical and nutritional value when the conditions were optimal. CAPP treatment induced significant changes in several chemical compositions. The total phenolic content was increased compared to control samples at some points of time during the 48 h to 96 h of pre-germination. GC-MS analysis revealed 13 identifiable compounds. It appeared likely that the biosyntheses of the 13 compounds in the pre-germinated seed samples, these being three simple phenolic compounds, two pyrans, one furan, one quinone, and six fatty acids, were accelerated in the plasma-treated groups. In case of legumes, the CAPP treatment of seeds, sprouts, and juvenile plants seems promising, as plasma treatment could

i. enhance the bioavailability of contained flavonoids, could further
ii. increase the flavonoid content, and/or could
iii. induce the selective modification of flavonoid profiles, for instance by triggering the increase in selected flavonol glycosides or even the formation of new derivatives.

In recent years, interest has grown in the utilization of legumes in other forms (e.g. flour, protein concentrate or isolate) rather than the whole seeds [149, 150]. Depending on the production process of alternative pea flour fractions, their techno-functional properties are limited [151]. For this purpose, a considerable amount of work has been accomplished in order to modify legume-based raw materials, intermediates and products while preserving their nutritional value. With regard to the post-harvest processing of legumes, the application of CAPP with its surface modifying effects, may significantly contribute to the bio-economic and resource efficient production of high-value legume-based intermediates and products. Plasma-induced surface modifications including the ablation of upper cell layers from hulled and unhulled pea surfaces, which were accompanied (Fig. 17) with the accelerated water uptake and increased swellability of the pea testa have been evidenced in former studies [152].

Thus, potential plasma applications may further aim at the targeted surface modification and the resulting functionalization of dry pea flours, powders or pellets. Results reported in literature demonstrate that cold plasma can be successfully applied in order to selectively modify the structure of proteins, starches and other biopolymers and therefore, improve their functionality (see section 1.2.2). Hence, this offers the opportunity to use plasma-treated food components as an ingredient in different formulated food to express targeted functionality. Up

to now, little literature is available dealing with this application-oriented use of plasma. Bahrami *et al.* [153] investigated the potential of CAPP to change the chemical composition of wheat flour through radical induced and ozone propagated oxidation and thereby, its applicability to change the functionality of the flour. As the oxidation of wheat flour proteins is believed to directly impact a range of functional properties, the oxidative changes in the protein may modify their molecular weights and solubility [154] and thus, their interactions with water and their ability to form a gluten network. The authors selected low treatment level in order to minimize any secondary effects, such as the formation of oxidation products that might be aroma active.

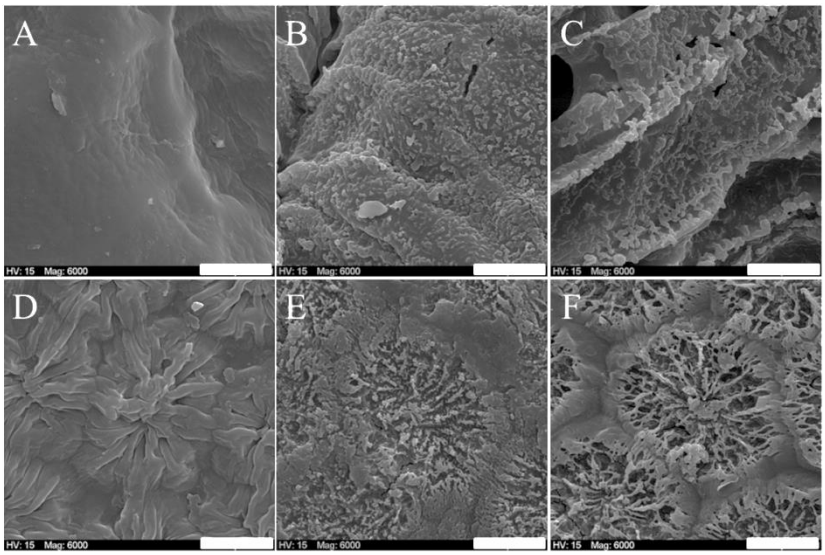

Figure 17: Scanning electron micrographs of morphological outer surface of hulled peas (A-C) and pea testa (D-F) prior to (A and D) and following plasma treatment for 5 min (B and E) and 15 min (C and F), respectively. Scale bars 5 µm, adapted from Bußler et al. [152].

A shift towards higher molecular weights in the protein profile was evidenced at higher energy inputs. The authors expected this plasma-induced effect to cause an increase in the strength of dough made from the flour, which indeed was the case, as small scale dough rheology tests revealed. As formerly mentioned, Misra, *et al.* [114] explored the possible effects of CAPP as a means to change the structural and functional properties on strong and weak wheat flours and attributed the voltage and treatment time-dependent increase in the viscoelasticity of the dough produced from the wheat flour to changes in the secondary structure of gluten proteins, as analyzed via FTIR spectroscopy. Another study conducted by Zou *et al.* [155] investigated the surface modification of starch in an argon glow discharge plasma. Changes were manifested in a loss of OH groups, which is probably due to the cross-linking of α-D-glucose units. Playing the dominant role in degradation reactions, surface proteins and proteinaceous matters were degraded most likely due to the impact of atomic oxygen [156]. In addition, a potential synergistic effect of nitric oxide contributing to the decomposition and minor roles for UV photons, OH radicals and metastable states of O_2 have been identified [157].

Also in related scientific fields a lot of work has been conducted regarding the plasma-induced effects on proteins, which evidenced that CAPP application induces modifications in

their secondary and tertiary structure. As the functionality of proteins is closely connected to their structure, plasma-induced effects consequently may offer an innovative approach to inhibit or modify proteins' functionality. For this purpose, the detailed knowledge on the raw material including composition, protein- and techno-functional properties, and in which way these specific material properties are affected by processing is an indispensable prerequisite for the tailored improvement of selected properties. Consequently, studies on the application of innovative technologies as alternatives to conventional treatments should include

i. the detailed characterization of the raw material used,
ii. the selection of proper process setup and process parameters,
iii. monitoring of the resulting plasma characteristics in order to achieve desired modifications, which need to be investigated using
iv. suitable analytical methods providing insights into possible underlying mechanisms.

To this end, selected raw material was analyzed in detail in order to identify appropriate objectives and methods from macroscopic to microscopic level (Fig. 18). Starting from evaluating the process-induced impact on quality attributes, investigations of changes in techno-functional properties need to be substantiated and further analyzed in order to gain knowledge on underlying mechanisms, which is precondition for the cross-value chain transfer of knowledge, as well as for the derivation of targeted applications.

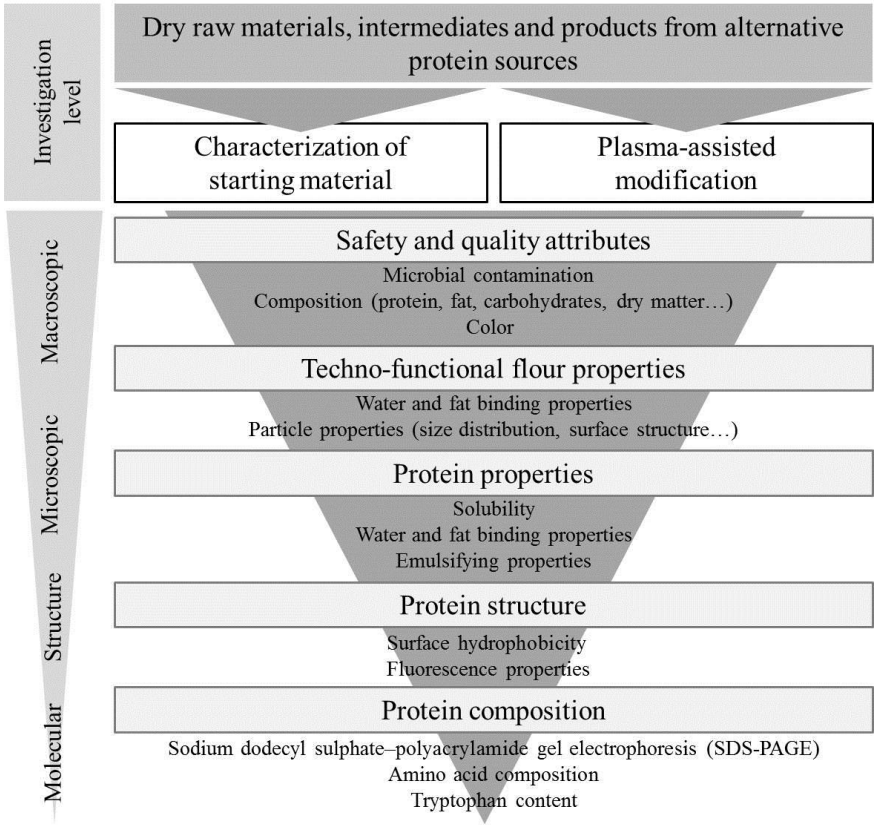

Figure 18: Schematic depiction of the top-down approach in the utilization of analytical methods providing insights into possible underlying plasma-induced mechanisms in protein-rich flours from macroscopic to molecular level.

As flours, protein intermediates and products are usually available in a dry form possessing limited functionality, the initial step in making proteins a functional ingredient is most likely hydration or solvation. Further, several other functional properties are dependent on the protein's solubility, thus, making this attribute extremely important for the overall protein applicability [158]. Swelling, water holding, retention and binding, as well as hydration capacity and water adsorption refer to the ability of protein molecules to interact with water. Incorporation of water in turn contributes to texture, juiciness and mouth feeling of a product and therefore, to its acceptability by the consumer.

Protein solubility is influenced by the balance of hydrophobic and hydrophilic amino acids on its surface. Since a hydrophilic protein surface causes good water solubility, charged amino acids play the most important role in keeping the protein soluble. CAPP, with its surface modifying properties, therefore, offers a promising approach regarding the modification of hydrophobicity-hydrophilicity-balance on the surface of proteins including possible cross-linking, hydrolysis and amino acid side chain reactions, which are usually induced by enzymatic or chemical modifications. Plasma treatment, in contrast, may be capable of inducing desired protein modifications without the addition of enzymes, chemicals and water. With regard to the plasma-induced effects on single food components studied in model systems, the CAPP technology may offer an innovative approach for the modification of techno-functional protein-properties of protein-rich, starch-rich, and fiber-rich fractions, as well as of a protein isolate from grain pea (*P. sativum* 'Salamanca') representing selected examples for complex dry food matrices. The detailed characterization of the starting material is a prerequisite for the specification of reasonable process conditions. Further, analysis offering insights into underlying mechanisms are a necessary precondition for the cross-value chain transfer of knowledge, as well as for the derivation of tailor-made applications of the CAPP technology in processing of food.

This part of the thesis aims at investigating the integration of the CAPP technology into the value-added chain of peas by

i. studying the use of plasma-induced effects on plant secondary metabolites in a targeted manner in order to selectively modify ingredient profiles in juvenile pea seedlings by using the CAPP technology as a kind of stress-inducing treatment (Section 4.1.1),

ii. characterizing the raw material in detail including composition, protein- and techno-functional properties and in which ways these specific material properties are affected during production and further processing (Section 4.1.2),

iii. investigating the use of plasma-induced surface modifications as a tool for the targeted modification of proteins' tertiary and secondary structure and thus, their functionality in dry raw materials, intermediates and products from peas as an alternative plant protein source (Section 4.1.3).

4.1.1 Impact of cold atmospheric pressure plasma on physiology and flavonol glycoside profile of peas (*Pisum sativum* ‘Salamanca’)

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Impact of cold atmospheric pressure plasma on physiology and flavonol glycoside profile of peas (*Pisum sativum* 'Salamanca')

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ABSTRACT

Application of plasma is well-established in various industrial processes; its use has also been suggested as an innovative technology in the food sector. Besides the ability to inactivate undesirable microorganisms on heat-sensitive foods, cold atmospheric pressure plasma (CAPP) may also modify and intensify the secondary metabolism in agricultural plant produces along the whole value-added chain. This is because CAPP provides a source of reactive oxygen and nitrogen species and specific UV radiation.

The objective of this study was to determine the effects of CAPP treatment on the flavonol glycoside profile of pea seedlings (*Pisum sativum* 'Salamanca'), while considering the potential impact on their metabolic activity in different growth stages. Pea seeds, sprouts, and seedlings were exposed to semi-direct CAPP using a dielectric barrier discharge device with air as the process gas. Applying voltages between 6 and 12 kV_{pp} at a frequency of 3.0 kHz resulted in optical emission spectra dominated by UV-B and UV-C radiation. The specific energy densities were monitored upon varying voltages and treatment times.

Exposing swollen pea seeds to plasma (9 kV_{pp}) between 1 and 10 min increased germination rate and dry matter content but decreased growth rate. Non-acylated and monoacylated triglycosides of quercetin and kaempferol dominated the flavonol glycoside profile, quercetin-3-*O*-*p*-coumaroyl-triglucoside being the main flavonoid glycoside. In 15 d-old pea seedlings, the concentration of flavonoid glycosides was dose-dependently decreased after two CAPP treatments compared to none or three treatments. Furthermore, photosynthetic efficiency of treated pea sprouts and seedlings declined potentially indicating a negative effect of CAPP treatment on plant metabolism. The responses of pea tissues greatly depended on time point and duration of CAPP treatments. This study represents a first step towards the implementation of the CAPP technology for a targeted modification of valuable secondary plant metabolites during post-harvest chain of agricultural produces.

1. Introduction

Cold atmospheric pressure plasma (CAPP) is well-established in different industrial applications. Besides the application of CAPP for processing of e.g., circuit boards and for ultra-fine cleaning of surfaces, its use is also suggested for the food sector. In initial laboratory-scale test series, mainly the possibilities of inactivating undesirable microorganisms on heat-sensitive foods such as fresh fruits and vegetables, meat and eggs have been studied (Schlüter et al., 2013). Moreover, the application of CAPP may offer a promising though still only marginally applied innovative technology along the whole value-added chain of plant-based produces.

It is well known that CAPP offers a source of reactive oxygen (ROS; e.g., atomic oxygen (O), ozone (O_3), hydroxyl radical (OH•)) and nitrogen species (RNS; e.g., N_2, NO, NO_2, nitric oxide radical (NO•)) as well as UV-A and UV-B radiation (Laroussi & Leipold, 2004). Thus, post-harvest application of CAPP on plant materials may be used as a targeted tool for the stress-induced modification and intensification of plants' secondary metabolism and, hence, concluding for the possibility of eliciting flavonoid synthesis without damaging the plant tissue (Baier et al., 2013).

To maximize the bio-economic use and to optimize the processing of raw materials, extensive research has been carried out in recent years. Those studies aimed at evaluating the effects of a multitude of various pre-harvest and post-harvest technologies including various kinds of radiation.

Representing one of the most important grain legumes (Adsule, Lawande, & Kadam, 1989), grain pea (*Pisum sativum*) is a major source

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of plant proteins, fibers, starch, and bioactive compounds. In particular, germinated grain legumes are excellent sources of dietary proteins and other nutrients, and contain non-acylated and monoacylated triglycosides of quercetin and kaempferol (Santos, Oliveira, Ibánez, & Herrero, 2014; Weissenböck, Hedrich, & Sachs, 1986). In the post-harvest chain of legumes, germination is key step for improving their nutritional quality and functionality (Gawlik-Dziki, Świeca, & Sugier, 2012; Ghavidel & Prakash, 2007, Świeca, Baraniak, & Gawlik-Dziki, 2013). In numerous studies, biotic and abiotic stresses as well as chemical and physical elicitors were used to effectively increase the biosynthesis of secondary metabolites (Gawlik-Dziki et al., 2012a; Ghavidel & Prakash, 2007, Randhir & Shetty, 2003; Zhao, Lawrence, & Verpoorte, 2005). It is well known that flavonoids are significantly involved in the stress responses of plants (Fujita et al., 2006; Khattak et al., 2007; McCune & Johns, 2007; Shetty, 2004). Due to their potential cardioprotective and anti-carcinogenic effects (Cook & Samman, 1996; Kris-Etherton et al., 2002; Trumbeckaite et al., 2006), flavonoids have gained considerable interest in recent years. In plants, flavonoids partially act as shielding components against excessive radiation both in the photosynthetically active and in the UV wavelength ranges. As effective antioxidants, they also play an important role in the plants' defense systems e.g., against reactive oxygen species (ROS) (Edreva, 2005).

So far, investigations on the impact of CAPP treatment on fresh fruits and vegetables are mainly limited to the assessment of external quality parameters such as appearance (Bermúdez-Aguirre, Wemlinger, Pedrow, Barbosa-Cánovas, & Garcia-Perez, 2013; Wang et al., 2012), metabolic activity (Tappi et al., 2014), fruit firmness (Misra et al., 2014), photosynthetic efficiency (Baier et al., 2013, 2014), and plasma–surface interactions (Grzegorzewski, Ehlbeck, Schlüter, Kroh, & Rohn, 2011). However, the potential mechanisms of CAPP on plant metabolism during storage are still poorly understood and investigations on the CAPP effects on protective secondary plant metabolites are scarce.

In the present study, a SDBD was used to treat seeds, sprouts, and seedlings of peas to investigate whether the application of CAPP to growing plants is capable of stimulating the increased synthesis of valuable plant components. Therefore, plasma-induced changes of the flavonoid glycoside profile of the pea seedlings were analyzed. In order to elucidate plasma-induced effects on the plant cell tissue, temperature profiles were recorded and the spectral composition of the plasma gas was characterized. Further, the effects of plasma exposure time on seed germination rates, as well as growth rates and dry matter contents of seedlings were analyzed.

2. Materials and methods

2.1. Plant materials

Dry grain peas (*P. sativum* 'Salamanca'; Norddeutsche Pflanzenzucht, Hans Georg Lembke GmbH, Hohenlieth, Germany) were soaked in tap water (1:4 w/w) at 23 °C for 22 h. For germination, 15 soaked pea seeds each were placed on Petri dishes (d = 1 cm) filled with 15 g of water-saturated perlite (Knauf Perlite GmbH, Dortmund, Germany) as growth medium. During the experiment, the Petri dishes were kept in a growth chamber (Bioline 1014, Weiss Umwelttechnik GmbH, Reiskirchen, Germany) at 23 °C day and 18 °C night temperatures, a relative humidity of 70% and a photosynthetic active photon fluence rate (PFR) of approx. 400 μmol m^{-2} s^{-1} (HQI-T Powerstar, 400 W, Osram GmbH, München, Germany) at a 14/10-h photo- and thermo-period. Plants were watered with 15 mL of tap water every 12 h.

Germination rate, determined 24 and 48 h after sowing, was calculated as percentages of the ratio (N_t/N_0) of the number of sown pea seeds (N_0) and that of pea seeds (N_t) germinated after the respective time (i.e., t = 24 h and t = 48 h). Growth rate of pea seedlings was determined as the length of the stems after 16 d. At the same time, dry matter content (DMC) of fresh samples and after exposure to CAPP was determined by weighing them before (fresh mass, FM) and after freeze-drying (dry mass, DM). For these measurements, pea seedlings were gently taken from the growing medium, adhering perlite was removed and the stem was severed from the roots and endosperms. The above-ground part of the stem was used for further analysis.

2.2. Cold atmospheric pressure plasma treatment

For cold atmospheric pressure plasma (CAPP) treatments of the plant material, a surface dielectric-barrier air-discharge (SDBD) system similar to that described by Oehmigen et al. (2010) was used. The SDBD plasma source consists of an array of 7 concentric ring-shaped electrodes (85 mm outer diameter) embedded in a 1.5 mm thick epoxy–glass bulk material and mounted into the upper shell of a Petri dish (90 mm diameter). Plasma treatments were performed in a cylindrical reaction chamber (15 cm height and 10 cm inner diameter), which surrounds the sample holder mounted on a height adjustable rotary shaker (JD 20, JVM Antriebe, Jöst, Dülmen, Germany). The SDBD plasma source was screwed in the cover of the reaction chamber assuring a constant installation position during the entire tests (Fig. 1). In order to prevent interactions between plasma-immanent species and ambient atmosphere, the airtight treatment chamber was filled with ambient air and hermetically sealed during plasma ignition. CAPP was generated at the surface of the dielectric epoxy glass by applying sinusoidal voltages of 6 to 12 kV_{pp} (during the process optimization steps) and 9 kV_{pp} (during the following experiments) at a frequency of 3.0 kHz delivered by a commercial function/arbitrary waveform generator (max. 20 MHz, DG1022, Rigol, Puchheim, Germany). The actual voltage was controlled via a built-in two channel digital storage oscilloscope (max. 500 MHz, TDS 2001C, Tektranx, Beaverton, USA).

For all trials, CAPP treatment of each of the 15 soaked pea seeds was conducted in an empty Petri dish, which was fixed on the holder of the shaker under the plasma source at a distance of 12 mm. Due to inhomogeneity in shape and size of the seeds, and height of the sprouts and seedlings in the following experiments, the distance of the plasma source was always adjusted to the bottom of the Petri dish as a reference point while ensuring a minimum distance of 20 mm of the uppermost leaves to the plasma source. Pea seeds were agitated continuously on the rotary shaker during the treatment. In an initial test series, exposure of the seeds to CAPP was varied between 1 and 10 min. Based on the results obtained from these trials, the duration of the plasma treatment in subsequent experiments was set to 5 min for seeds and sprouts and was varied between 2.5 and 10 min for seedlings.

Immediately after CAPP treatment, the entire 15 pea seeds per sample were transferred into prepared wet perlite-filled Petri dishes and dark acclimated for 10 min for subsequent chlorophyll fluorescence image analysis. For CAPP treatment of 8 d old pea sprouts, samples were placed in Petri dishes under the plasma source. The distance from the plasma source to the bottom of the Petri dish was adjusted to 30 mm. The same procedure was performed on 15 d old seedlings with the distance between the plasma source and the bottom of the Petri dish adjusted to 50 mm (Fig. 1). All the treatments were conducted in triplicate.

2.3. Determination of temperature profiles

During CAPP applications to the plant material, temperature increase in the reaction chamber was measured with a fiberglass-encased optic thermocouple (K-type) inserted through an optional opening. The thermocouple was positioned on the surface of the wetted perlite. At a frequency of 0.1 Hz, the results were recorded with a USB data acquisition system (Personal Daq/56, SynoTECH, Hückelhofen, Germany) and the DASYLab 13.0 software (Measurement Computing, Norton, USA) for 10 min. An initial temperature of 21 °C (±0.5 °C) was ensured by pre-tempering of the perlite containing Petri dishes in

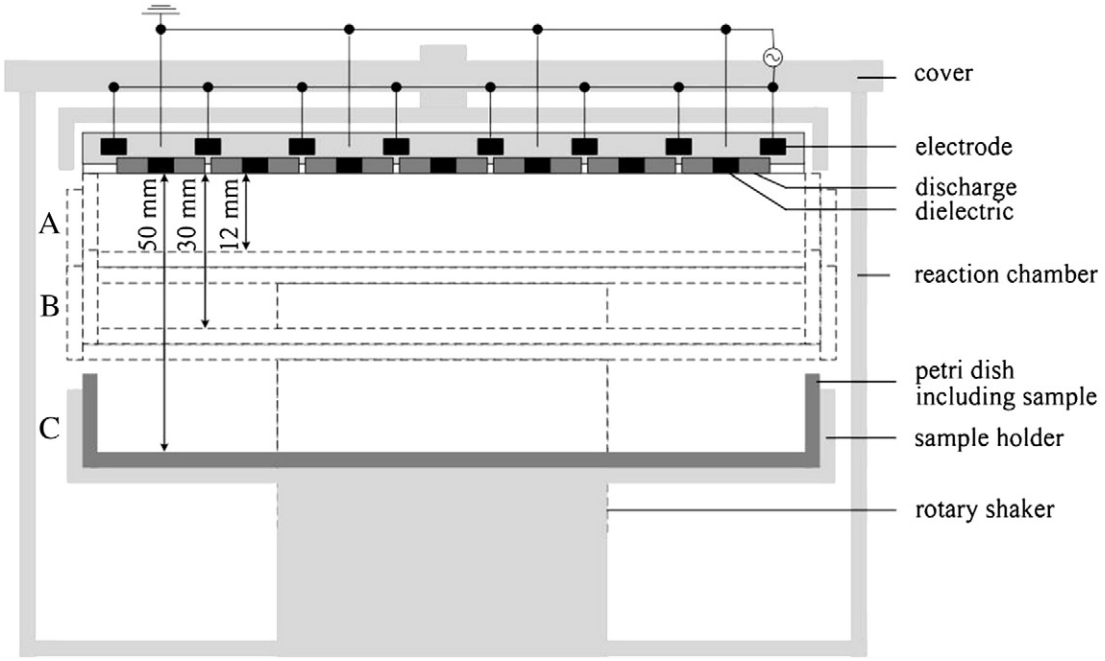

Fig. 1. Experimental set-up of the cold atmospheric pressure surface dielectric barrier discharge plasma device for the treatment of pea seeds (A), sprouts (B) and seedlings (C).

an incubator. In order to investigate whether the sample surface temperature exceeds the temperature in the treatment chamber, the maximum thermal load of the sample surface was measured as described above by using single leafs from peas fixed in a sample holder at a distance of 20 mm to the plasma source.

2.4. UV component and optical emission spectroscopy

The total amount of UV radiation of the plasma source was analyzed using control strips (UV-Tec Messtechnik GmbH, Bergisch Gladbach, Germany) with a measuring range from 5 to 60 mJ cm^{-2}. To monitor the contribution of the respective UV components at voltages from 6 to 12 kV_{pp}, emission spectra of the SDBD plasma were scanned 500 times (integration time 1 ms) at a range of 185 to 850 nm using a Black-Comet UV–vis Spectrometer (StellarNet Inc., Tampa, USA). The spectrometer was equipped with a F400-UV–vis-SR fiber optic, positioned under the surface of the plasma source at a distance of 20 mm in order to detect the plasma composition at the point where the first plasma–product interactions occur (uppermost leaves of sprouts and seedlings) and to ensure that the measurement was not distorted by shadow effects caused by overlapping leaves. The average of the recorded spectra was then base-line corrected and normalized using MATLAB (version 7.12, R2011a, The MathWorks, Inc., Natick, USA). The peak positions were compared with the data of the NIST Atomic Spectra Database (version 5.0; Kramida et al., 2012). Furthermore, the qualitative formation of ozone was detected by the use of a single gas detector (GasAlert Extreme, BW Technologies, Calgary, Canada).

2.5. Chlorophyll fluorescence imaging

Chlorophyll fluorescence imaging (CFI) was performed using a modular system (FluorCAM 700MF, PSI, Brno, Czech Republic), which measures sequences of fluorescence images with a user defined timing of set points, measurement intervals and irradiance (Herppich, 2002; Herppich, Foerster, Zeymer, Geyer, & Schlüter, 2012; Nedbal, Trtilek, & Herppich, 2000). As described in detail elsewhere (Baier et al., 2013; Schlüter, Foerster, Geyer, Knorr, & Herppich, 2009), the initial basic fluorescence (F_0) was induced by weak, non-actinic measuring-light pulses of two sets of 345 super-bright orange light emitting diodes (λ_{max} = 620 nm). Maximum fluorescence (F_m) was excited by a short-term (1 s) saturation light pulse (max. 2500 μmol photons m^{-2} s^{-1}), generated by a halogen lamp (250 W) equipped with an electronically controlled shutter. Fluorescence images were recorded by a CCD camera (12-bit, 512 × 512 pixels; maximum frame rate 50 images s^{-1}) equipped with an F1.2/2.8-6 mm objective and a short-pass filter system (high pass 695 nm, low pass 780 nm) synchronously with the weak, non-actinic measuring-light pulses. The system was controlled by a Windows XP compatible software (FluorCAM 6, PSI, Brno, Czech Republic).

All measurements were performed after pre-darkening seeds, sprouts, and seedlings for 10 min (von Willert, Matyssek, & Herppich, 1995). Measurements were conducted directly after the application of CAPP and, again, after 24 h. The potential impact of the plasma treatment on the physiological activity of pea samples was assessed by evaluating the maximum photon yield of electron transport through photosystem II (F_v/F_m; $F_v = F_m - F_0$). This parameter is a valuable tool to determine both capacity and stability of photosynthesis (Björkman & Demmig, 1987; Krause & Weis, 1991; von Willert et al., 1995) and its response to biotic and abiotic constraints. F_v/F_m ranges between 0.84 in highly active plants and 0 in fully damaged (dead) tissues (Björkman & Demmig, 1987) and F_v/F_m values below 0.1 were neglected.

2.6. Extraction and HPLC–DAD–ESI-MS^n analysis of flavonoid glycosides

Following harvest, pea seedlings were immediately frozen in liquid nitrogen, lyophilized and ground. Flavonoids were analyzed according to Neugart et al. (2014) with slight modifications. The lyophilized powder (20 mg) was extracted with 600 μL of 60% aqueous methanol at 20 °C on a magnetic stirrer plate for 40 min, centrifuged at 4500 rpm at the same temperature for 10 min and the supernatant collected in a reaction tube. The process was repeated twice with 300 μL of 60% aqueous methanol, shaking for 20 min and 10 min, respectively, and centrifugation for 10 min, all at 20 °C. The supernatants were finally combined, the extract evaporated to dryness, suspended in 200 μL of 10% aqueous methanol and filtered through Corning® Costar® Spin-X® plastic centrifuge tube filters (Sigma Aldrich Chemical Co., St.

Louis, USA) for HPLC analysis. Each extraction was carried out in duplicate.

An Agilent 1100 series HPLC (Agilent Technologies GmbH, Waldbronn, Germany) consisting of degasser, binary pump, autosampler, column oven and photodiode array detector was used to determine hydroxycinnamic acid derivatives and glycosides of flavonols. A Supelco Ascentis® Express F5 column (150 mm × 4.6 mm, 5 μm, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) was used to separate the compounds at a 0.85 mL min^{-1} flow and a temperature of 25 °C. Eluent A was 0.5% acetic acid and eluent B was 100% acetonitrile. The gradient used for eluent B was 5–12% (0–3 min), 12–25% (3–46 min), 25–90% (46–49.5 min), 90% isocratic (49.5–52 min), 90–5% (52–52.7 min) and 5% isocratic (52.7–59 min). Hydroxycinnamic acids, acylated flavonol glycosides and non-acylated flavonol glycosides, respectively, were determined at wavelengths of 320, 330 and 370 nm. The hydroxycinnamic acid derivatives and glycosides of flavonols were identified according to Schmidt et al. (2010) by HPLC–DAD–ESI-MS^n using an Agilent 1100 series ion trap mass spectrometer in negative ionization mode. Nitrogen was used as the dry gas (10 L min^{-1}, 325 °C) in addition to nebulizer gas (40 psi) with a capillary voltage of −3500 V. Helium was used as the collision gas in the ion trap. The mass optimization for the ion optics of the mass spectrometer was performed for quercetin *m/z* 301. The MS^n experiments were performed in auto up to MS^3 in a scan from *m/z* 200–2000. Chlorogenic acid, quercetin-3-*O*-glucoside and kaempferol-3-*O*-glucoside (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were used as standards for external calibration curves.

2.7. Statistical analysis

All data were statistically analyzed (ANOVA) with Statistica™ for Windows™ (version 9.0, Statsoft Inc., Tulsa, Okla.). Significant differences between means were determined by Turkey's HSD test ($p < 0.05$). In the figures, the mean variability of data was indicated by the standard deviation.

3. Results and discussion

3.1. Characterization of the SDBD plasma source

To get insight into the composition of the plasma applied and how it is related to the chosen processing parameters, the emission spectra of the SDBD plasma source were analyzed. Especially for the treatment of plant material, UV-composition and energy flux of the applied irradiation represent essential characteristics for process control. The obtained emission spectra were dominated by UV-radiation (200–400 nm), whereas only a few peaks were detected in the visible light range (Fig. 2A). An UV component of 15 mJ cm^{-2} could be detected within 60 s of plasma exposure corresponding to an energy flux of 0.25 mW cm^{-2}. No UV-C radiation below 280 nm was measured (NO_y system) (Fig. 2B), which might have the ability to damage DNA. These findings are in accordance with those obtained by Laroussi and Leipold (2004). The UV-B spectrum was characterized by molecular bands of the third (296.5 nm), fourth (315.5 nm), and second positive systems of N_2 (336.5, 354, 357, 372, 374.5 and 379.5 nm). In contrast to the results presented by Laroussi and Leipold (2004), the presence of OH• could not be evidenced in relevant amounts during these trails. This may be due to their short life (Shimmura et al., 1999). UV-A and UV-B radiation may also damage cells due to the generation of peroxide radicals and resulting oxidation reactions (Koutchma, Forney, & Moraru, 2009). Irrespective of the applied voltage (Fig. 2B; range 6 kV_{pp} to 12 kV_{pp}) the resulting UV spectra were dominated by UV-A radiation (relative intensities 0.70 to 0.82, based on the total UV emission), while UV-B and UV-C radiation contributed to similar degrees (7 to 18%). Exposure to SDBD plasma at a voltage set to 9 kV_{pp} for up to 10 min in all following experiments implied a time-dependent application of UV (UV-C, UV-B and UV-A) radiation with a maximum energy flux of 6 (0.55, 1.07 and 4.38) kJ m^{-2} (Table 1).

Furthermore, the formation of ozone during CAPP treatment was qualitatively demonstrated as reported in literature (Jayasena et al., 2014; Kalghatgi, Fridman, & Azizkhan-Clifford, 2012). Ozone is a powerful oxidizer for both organic and inorganic substances and is typically formed around high-powered electrical equipment where sparking is evident. Due to its comparatively long lifetime and high oxidation potential, ozone is considered as one of the chemically most stable and active species generated in DBD plasma setups. It was also classified GRAS (generally recognized as safe) by the US Food and Drug Administration when being used as a direct additive in food (FDA, 2001; Rice & Graham, 2001). The additional reactive species in the DBD plasma-produced gas phase were superoxide, hydrogen peroxide, singlet oxygen, nitric oxide, electrons, and positive ions, as also identified in other studies (Fridman, 2008; Fridman, Chirokov, & Gutsol, 2005; Kennedy & Fridman, 2004).

The recorded temperature profiles confirmed that generation voltages below 10 kV_{pp} prevented a negative thermal impact of the SDBD based CAPP (Fig. 3) and that sample surface temperatures did not exceed the temperature in the treatment chamber (data not shown). The initial temperature (approx. 20.5 °C) of samples exposed in the

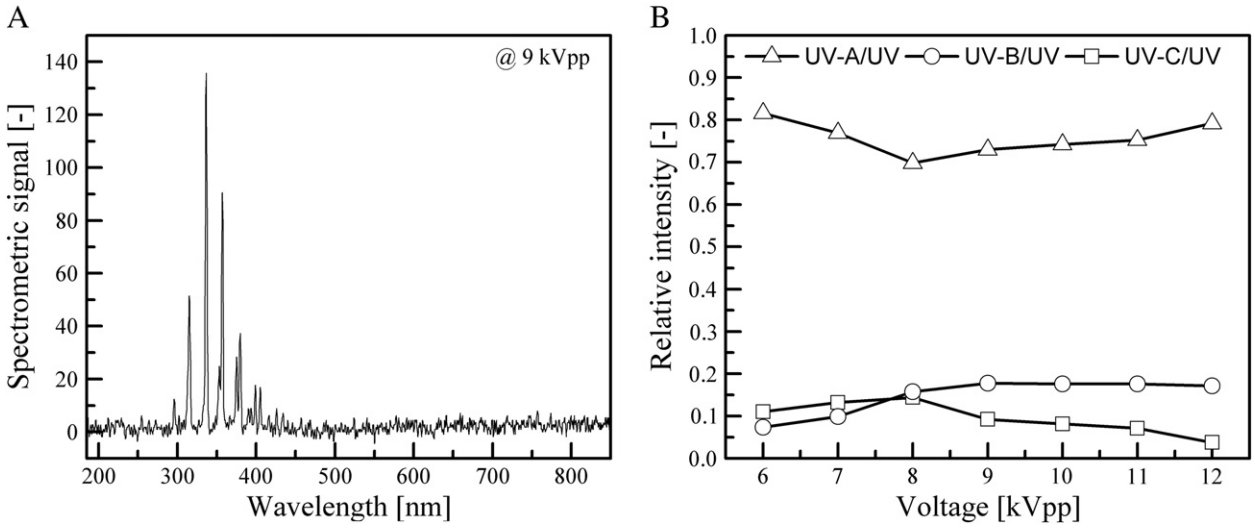

Fig. 2. (A) Optical emission spectra of the plasma generated with the SDBD device in air at an applied voltage of 9 kV_{pp} (3.0 kHz, 20 mm distance from the plasma source). (B) Relative composition of UV-radiation (UV-A, UV-B and UV-C) at various voltages ranging from 6 to 12 kV_{pp} (3.0 kHz, 20 mm distance from the plasma source).

Table 1
Maximum doses of UV-C, UV-B, UV-C, and total UV emission applied to pea sprouts and seedlings under varying plasma exposure times at a distance of 20 mm from the plasma source (9 kV_{pp}, 3.0 kHz). All differences in means are statistically significant ($p < 0.05$).

Exposure time [min]	UV-C [kJ m^{-2}]	UV-B [kJ m^{-2}]	UV-A [kJ m^{-2}]	UV total [kJ m^{-2}]
0	0	0	0	0
2.5	0.14	0.27	1.09	1.50
5	**0.28**	**0.53**	**2.19**	**3.00**
10	0.55	1.07	4.38	6.00

treatment chamber only slightly increased to 21 °C during CAPP treatment at a voltage of 9 kV_{pp} for 10 min. Increasing the voltage to 12 kV_{pp} increased temperature in the treatment chamber to a maximum of approx. 25 °C.

3.2. Germination, growth and dry matter content

After adjustment of the pea seeds for 24 and 48 h, 22% and 44%, respectively, of the untreated seeds germinated (Fig. 4A). Plasma exposure for up to 2 min did not affect germination rates; whereas plasma exposure times beyond 2 min substantially increased them. When seeds were CAPP-treated for 5 and 10 min, germination after 24 h increased to 42% and 50%, respectively. This effect partially diminished 48 h after the treatment but was still evident for treatment times of 3 and 5 min. With a 5 min CAPP exposure, germination rates of pea seeds reached a maximum (approx. 65%) after 48 h. Similar effects have been reported for soybean (Filatova et al., 2011; Volin, Denes, Young, & Park, 2000).

In contrast, exposure to CAPP negatively influenced growth as indicated by reduced stem length of 16 d-old pea seedlings (Fig. 4B). Exposure to CAPP for 1 and 2 min decreased stem length by 25.0% and 35.3%, respectively. Increasing duration of CAPP treatment to 3 min decreased stem growth further by 45.6%. Extending treatment to 5 and 10 min, however, did not show any extra effect on length growth. In addition, CAPP treatment had only a very minor effect on the dry matter content of seedlings, which slightly (8.2%) increased from 19.7 mg g^{-1} in controls to a maximum of 21.5 mg g^{-1} in plants exposed to CAPP for 10 min.

The impact of CAPP treatment on seed germination and growth of seedlings may result from various interactions between reactive species of the plasma and the surface and/or the histological structure, and the biochemical composition of the pea seeds. However, the relevant, actually CAPP-affected process(es) out of those many mechanisms,

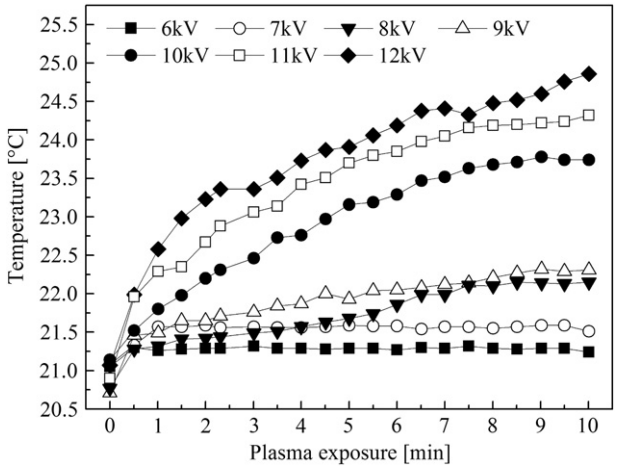

Fig. 3. Temperature in the reaction chamber during plasma treatment in air, varying the applied voltages from 6 to 12 kV_{pp} (3.0 kHz).

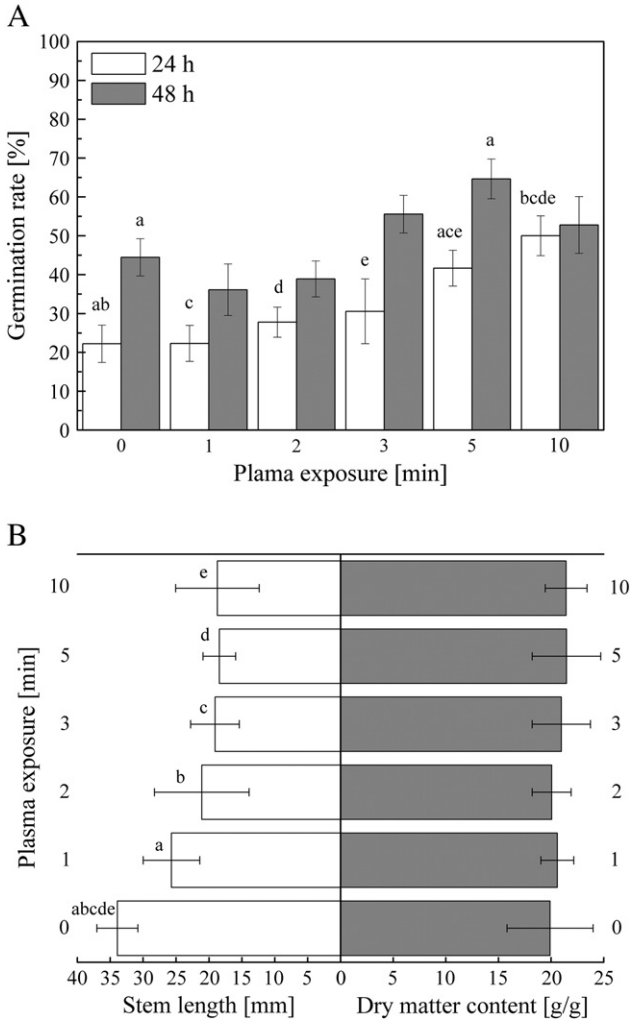

Fig. 4. (A) Germination rates of pea seeds directly after exposure to CAPP (9 kV_{pp}, 3.0 kHz) for 0 to 10 min and after adaptation for 24 h. (B) Resulting stem lengths and dry matter contents after growth for 16 d. Different letters indicate significant ($p < 0.05$) differences between means.

diversely involved in germination and growth, remained unidentified. In this study, the effects obtained may be caused by a combination of various factors. Among others, the inactivation of enzymes as well as the decomposition or modification of endogenous substances such as gibberellic acid often occurring after plasma treatments (Surowsky, Fischer, Schlüter, & Knorr, 2013; Tappi et al., 2014) may potentially decrease growth rates. In some cases, seed testa-imposed dormancy may be alleviated by ROS, which can oxidize the phenolic compounds present in the seed testa. This may allow improved oxygenation of the embryo (Fontaine, Huault, Pavis, & Billard, 1994; Ogawa & Iwabuchi, 2001). Alternatively, it can cause cracking in the coat of hard seeds, thus facilitating imbibition (Chien & Lin, 1994, Volin et al., 2000). ROS may also stimulate germination of dormant seeds (Fontaine et al., 1994; Ogawa & Iwabuchi, 2001; Wang, Heimovaara-Dijkstra, & Van Duijn, 1995; Wang et al., 1998) by activating the oxidative pentose phosphate pathway (Fontaine et al., 1994; Hendricks & Taylorson, 1975).

Additionally, the UV radiation emitted by the SDBD plasma source has to be taken into consideration since it was reported that UV irradiation can accelerate seed germination accompanied by a retarded seedlings growth (Noble, 2002). Decreased growth of sprouts may also be attributed to the presence of ozone. Ozone did not affect seed germination but let to minor visible injuries and significantly decreased growth

in a time and ozone concentration related manner (Hogsett, Plocher, Wildman, Tingey, & Bennett, 1985).

3.3. Photosynthetic efficiency and dry matter content

As expected, F_v/F_m in pea seed was below 0.1 and is most likely attributed to a purely physical effect due to the contained chlorophyll in the tissue. During the next 7 d, photosynthetic efficiency of pea sprouts from untreated seeds increased by 51.9% (Table 2). In seedlings, grown from seeds, which had been CAPP-exposed for 5 min, maximum photochemical efficiency of photosystem II (PSII) was significantly lowered by 7.6% compared to plants raised from untreated seeds. Exposing these sprouts of untreated seeds to CAPP for 5 min significantly lowered F_v/F_m by 13.9%. Furthermore, exposing sprouts grown from plasma-treated seeds (CAPP for 5 min) significantly decreased this parameter by 37.9%.

Following 24 h relaxation of the sprouts, the maximum photochemical efficiency of PSII increased by 10.8% in untreated sprouts grown from untreated seeds and was marginally lower in seedlings of the CAPP-treated seeds. In comparison, in plasma treated sprouts from the untreated seeds, F_v/F_m increased by 38.1%, while it increased by 14.2% in spouts that had been exposed to CAPP in the seed and in the sprout stage. Consequently, the impact of CAPP treatment of seeds is almost negligible when compared to the CAPP-induced effects by treatment of the sprouts.

The sensitivity of sprouts to CAPP exposure increased with continued growth. Moreover, a significant reduction of F_v/F_m was induced by double application of CAPP whereby the sprouts recovered quickly within 24 h. During the next 7 days of growth, photosynthetic efficiency of the pea seedlings only slightly increased by 2.3% for plants raised from untreated seeds assuming that photosynthetic efficiency continued to rise at this growth stage. In contrast, for pea seedlings exposed to CAPP as seeds, an increase in photosynthetic efficiency by 13.4% was determined, demonstrating that exposing the pea seeds to CAPP may even positively influence photosynthetic efficiency. Pea seedlings, which had been exposed to CAPP as sprouts were characterized by an increased photosynthetic efficiency, whereas double CAPP treatment of seeds and sprouts lowered F_v/F_m of 15 d-old pea seedlings. This, again, reflects an increased sensitivity of the sprouts towards exposure to CAPP. Varying durations of plasma exposure from 0 to 10 min largely influenced the physiological fitness of all seedlings. Photosynthetic efficiency of seedlings, which had never been exposed to CAPP during growth was drastically reduced and greatly diminished compared to the untreated sample even after adapting for 24 h. In comparison, repeated treatments of seedlings raised from CAPP-exposed seeds led to a likewise strong but less markedly decrease in physiological fitness. This was further decreased by re-treating seedlings exposed-CAPP as sprouts. After 24 h dark storage, seedlings, which had only been treated as sprouts partially recovered. Seedlings, developed from CAPP-treated seeds less pronouncedly responded to plasma treatment as sprouts. In this case, however, recovery and partial acclimation to plasma-induced stress was observed after 24 h. These effects were accompanied by an increase in dry matter content. Although this increase was statistically not significant, the tendency may, nevertheless, be attributed to the CAPP-induced decrease in physiological fitness of the seedlings as reflected by the decrease in F_v/F_m.

Due to their complex structure defending plants from environmental stress, it is rather unlikely that ROS are able to penetrate from outside into the plant tissue. But in plants, ROS are also naturally produced in a great variety of metabolic reactions that may be stimulated by plasma-induced stress. As the oxidation–reduction cascades can provide the driving force for metabolism and redox signals, highly influencing the biosynthetic activities in seeds, the induced synthesis of ROS poses an intrinsic impact to plant tissues due to the susceptibility to oxidative damage of many components within the photosystems (Foyer, Lopez-Delgado, Dat, & Scott, 1997). ROS have been identified as a key component in at least the signaling pathway(s) leading to the down-regulation of photosynthesis (Jordan, James, Strid, & Anthony, 1994; Mirecki & Teramura, 1984; Zanocco, Pavez, Videla, & Lissi, 1989).

Numerous studies demonstrated that PSII is the component of the thylakoid membrane most sensitive to exposure to UV-B radiation (Iwanzik et al., 1983; Noorundeen & Kulandaivelu, 1982; Renger et al., 1986). Bornman and Teramura (1993) suggested that, during the early stages of development, seedlings may be particularly sensitive to UV-B. In addition, inhibition of photosynthesis in UV-B irradiated fully expanded leaves was more pronounced (Teramura & Caldwell, 1981). These effects may possibly explain the acclimatization to CAPP treatment, in particular, the recovery of peas exposed to the plasma in early growth stages. Acclimation may potentially result from reduced UV-B penetration into leaves due to the accumulation of UV-B absorbing

Table 2
Means (±sd; n = 45) of maximum photochemical efficiency (F_v/F_m) and dry matter content of pea seeds, sprouts and seedlings directly after the exposure to CAPP (A) and after 24 h relaxation (B) as treated over during a growth period of 15 days.

Exposure to CAPP [min] as			Growth stage				
			Sprouts		Seedlings		
Seeds	Sprouts	Seedlings	Photosynthetic efficiency F_v/F_m [−]				Dry matter [g/g]
			A	B	A	B	
0	0	0	$0.46^{A} \pm 0.07$	$0.55^{A*} \pm 0.07$	$0.57^{aA} \pm 0.03$	$0.63^{aA} \pm 0.05$	$19.7^{aA} \pm 4.1$
		2.5			$0.51^{aA} \pm 0.09$	$0.58^{aA} \pm 0.10$	$20.1^{bA} \pm 1.6$
		5			$0.03^{bAB} \pm 0.00$	$0.19^{bA*} \pm 0.10$	$21.4^{bA} \pm 2.8$
		10			$0.02^{bA} \pm 0.0$	$0.00^{cA} \pm 0.05$	$21.4^{bA} \pm 3.0$
	5	0	$0.35^{B} \pm 0.03$	$0.51^{A*} \pm 0.06$	$0.63^{aBD} \pm 0.05$	$0.84^{aBCD*} \pm 0.04$	$18.7^{aA} \pm 3.3$
		2.5			$0.53^{bAB} \pm 0.02$	$0.72^{bBCE*} \pm 0.05$	$23.9^{bAB} \pm 2.4$
		5			$0.44^{cA} \pm 0.06$	$0.61^{cB*} \pm 0.05$	$21.7^{bA} \pm 3.4$
		10			$0.35^{dBCD} \pm 0.00$	$0.51^{dBD*} \pm 0.04$	$22.0^{bA} \pm 3.1$
5	0	0	$0.40^{B} \pm 0.09$	$0.54^{A*} \pm 0.08$	$0.61^{aA} \pm 0.05$	$0.79^{aCD*} \pm 0.04$	$18.8^{aA} \pm 1.2$
		2.5			$0.46^{bBC} \pm 0.10$	$0.69^{bCF*} \pm 0.05$	$18.6^{aAC} \pm 3.5$
		5			$0.37^{bB} \pm 0.10$	$0.40^{cC} \pm 0.05$	$20.8^{bA} \pm 3.5$
		10			$0.24^{cC} \pm 0.05$	$0.32^{cC*} \pm 0.04$	$23.1^{bA} \pm 2.0$
	5	0	$0.15^{C} \pm 0.07$	$0.38^{B*} \pm 0.08$	$0.56^{aA} \pm 0.05$	$0.84^{aD*} \pm 0.03$	$17.4^{aA} \pm 1.7$
		2.5			$0.48^{bABD} \pm 0.06$	$0.83^{aD*} \pm 0.03$	$19.6^{aA} \pm 4.1$
		5			$0.47^{bC} \pm 0.07$	$0.76^{bD*} \pm 0.03$	$21.2^{bA} \pm 1.9$
		10			$0.35^{cD} \pm 0.04$	$0.58^{cD*} \pm 0.03$	$22.9^{bA} \pm 3.7$

Also given are the dry matter contents of seedlings. Different lower case characters indicate significant ($p < 0.05$) differences between means comparing F_v/F_m of plasma treated seedlings that had undergone equal plasma treatment as sprouts. Different upper case characters indicate significant ($p < 0.05$) differences between means comparing F_v/F_m of equally plasma treated seedlings that had undergone different plasma treatments as sprouts. Significant differences in means comparing F_v/F_m of seedlings directly after exposure to plasma (A) and after 24 h relaxation (B) are marked with *.

pigments such as flavonoids (Allen, 1998). This assumption could, however, not be confirmed by the results obtained in this study (see Section 3.4).

Furthermore, it was reported that ozone negatively affects a number of plant processes, including photosynthesis (Krupa, 1997). The damaging effects of ozone on various components of the light-harvesting complex in the chloroplasts were identified.

Finally, the effects, obtained in this study, cannot be traced to one specific cause but rather to a combination of effects induced by the diverse plasma-immanent species which can mutually act synergistically or antagonistically. However, a study of Ling et al. (2014) indicated the positive impact of cold plasma on the growth and yield of soybean.

3.4. Flavonol glycoside profile

In pea seedlings non-acylated and monoacylated triglycosides of quercetin and kaempferol were found (Tables 3 and 4). The acylated hydroxycinnamic acids are *p*-coumaroyl, feruloyl, and sinapoyl, all of them providing no catechol structure at the phenolic ring. Additionally, caffeic acid (containing a catechol structure at the phenolic ring) was acylated to a quercetin glycoside. The main flavonol glycoside of pea seedlings is quercetin-3-*O*-*p*-coumaroyl-triglucoside. These flavonoid glycosides of peas have already been reported before (Santos, Oliva-Teles, Delerue-Matos, & Oliveira, 2014; Santos et al., 2014; Weissenböck et al., 1986). According to the fragmentation spectra in Schmidt et al. (2010), all flavonoid glycosides have been tentatively identified as 3-*O*-triglucosides resulting from the specific loss of *m/z* 486 in MS^3.

Increasing exposure time to CAPP at day 15 tended to lower the concentrations of flavonoid glycosides in seedlings independently of whether peas were CAPP-treated in the seed or sprout stage or not (Table 3). Quercetin-3-*O*-*p*-coumaroyl-triglucoside decreased after two CAPP treatments (at the stages seed and seedling or at the stages sprout and seedling), while quercetin-3-*O*-feruloyl-triglucoside decreased after three CAPP treatments (as seed, sprout and seedling) only. Both quercetin glycosides are marked by containing hydroxycinnamic acids without the antioxidatively effective catechol structures. Plasma includes both ROS and UV radiation. It is generally accepted that plants accumulate phenolics in response to higher UV-B radiation due to the UV-B induced higher concentrations of ROS in the plant cells (Jansen, Hectors, O'Brien, Guisez, & Potters, 2008). The effect of extracellular ROS on flavonoid glycosides has not been addressed before. Additionally, the specific response of structurally different flavonoids to UV-B radiation or other abiotic factors is not yet discussed comprehensively. In kale, the kaempferol triglycosides were less affected by subsequent doses of UV-B than the corresponding kaempferol diglycosides and kaempferol tetraglycosides (Neugart et al., 2014). In contrast, pea seedlings comprise only triglycosides of quercetin and kaempferol. In accordance to kale these flavonol triglycosides in pea also seem to be less affected by CAPP exposure. Thus, the common fact that quercetin glycosides can be enhanced by UV-B (Agati, Cerovic, Pinelli, & Tattini, 2011; Goetz et al., 2010; Morales et al., 2010) cannot be transferred to CAPP treatments assuming either a distinct different effect of the ROS concentration in the plasma or a more diverse effect of the total UV dose (UV-A, B and C) of the plasma. Compared to environmental conditions, the UV-A and UV-B doses of the applied CAPP are low, but the dose of UV-C, which is a more efficient stressor is higher. In kale, caffeic acid monoacylated kaempferol triglucoside, but not ferulic acid and sinapic acid monoacylated kaempferol glycosides, was strongly enhanced by subsequent doses of moderate UV-B (Neugart et al., 2014). In the present study, flavonoid glycosides (acylated and non-acylated with hydroxycinnamic acids) tended to decrease. Moreover, irradiating mountain birch leaves with UV-B treatment of 6–10 kJ m^{-2} d^{-1} for several weeks, did not affect their quercetin glycoside contents (Anttila et al., 2010) because this mountain plant is highly adapted to high levels of UV radiation with corresponding higher concentrations of ROS. Pea seedlings seem to be well adapted to higher contents of ROS due to high concentrations of quercetin glycosides. However, an increased synthesis of flavonoid glycosides especially the acylated *p*-coumaric acid and the acylated ferulic acid as antioxidant may only be implemented in plant defense response after two or more plasma treatments, which consequently dose-dependently reduces their concentration after CAPP treatment.

In lamb's lettuce, concentrations of the two flavonoids luteolin and diosmetin increased by direct exposure to the afterglow of a radiofrequency driven discharge plasma for 2 min (Grzegorzewski, Rohn, Kroh, Geyer, & Schlüter, 2010; Grzegorzewski et al., 2011). However, in the present study, a single CAPP treatment of 2.5 to 10 min (0.27–1.07 kJ m^{-2} d^{-1} UV-B) had no effect on the flavonoid glycoside concentration of pea seedlings. In kale, a single dose of moderate UV-B radiation (0.5–2.00 kJ m^{-2} d^{-1}) led to the response of flavonoid glycosides dependent on their chemical structure. While quercetin glycosides and monoacylated kaempferol tetraglucosides decreased with a single dose of moderate UV-B, monoacylated kaempferol diglucosides increased significantly (Neugart et al., 2012). In pea seedlings, a low response of flavonoid glycosides was found at single doses of up to 0.55 kJ m^{-2} (UV-C), 1.07 kJ m^{-2} (UV-B) and 4.38 kJ m^{-2} (UV-A), assuming that pea seedlings might be protected against high concentrations

Table 3
Concentration of quercetin glycosides (ng g^{-1}) of pea seedlings after exposure to CAPP followed by 24 h adjustment. Different letters indicate significant ($p < 0.05$) differences between means.

Exposure to CAPP [min] as			Concentration of quercetin glycosides [ng g^{-1}]						
Seeds	Sprouts	Seedlings	Q-triglc	Q-3-caf-triglc	Q-3-cou-triglc	Q-3-sin-triglc	Q-3-fer-triglc	Q-3-cou-triglc (derivative)	Total QG
0	0	0	140 ± 18	62 ± 12	572 ± 194	92 ± 43	101 ± 11	96 ± 17	100 ± 283
		2.5	93 ± 32	73 ± 8	316 ± 35	60 ± 0	84 ± 7	73 ± 9	626 ± 84
		5	96 ± 44	78 ± 24	305 ± 154	77 ± 39	101 ± 43	87 ± 39	666 ± 319
		10	93 ± 17	75 ± 24	291 ± 96	73 ± 17	94 ± 19	85 ± 24	636 ± 173
	5	0	252 ± 115	142 ± 89	755^{b} ± 223	192 ± 91	198 ± 101	185 ± 102	158^{b} ± 630
		2.5	161 ± 56	123 ± 39	490^{b} ± 92	128 ± 42	153 ± 46	143 ± 48	148^{ab} ± 436
		5	98 ± 37	78 ± 18	314^{ab} ± 194	75 ± 20	96 ± 33	89 ± 29	672^{ab} ± 43
		10	79 ± 23	76 ± 17	203^{a} ± 103	70 ± 20	85 ± 24	79 ± 20	516^{a} ± 191
5	0	0	144 ± 115	147 ± 89	376^{ab} ± 137	122 ± 123	159 ± 127	147 ± 123	947 ± 626
		2.5	132 ± 29	75 ± 25	670^{b} ± 50	139 ± 24	142 ± 33	113 ± 28	111 ± 0.165
		5	85 ± 31	70 ± 18	298^{a} ± 196	75 ± 24	92 ± 35	83 ± 29	633 ± 314
		10	67 ± 32	60 ± 32	258^{a} ± 120	48 ± 19	67 ± 26	59 ± 24	499 ± 220
	5	0	133 ± 27	71 ± 12	573 ± 176	101 ± 39	128^{b} ± 24	104 ± 24	130 ± 348
		2.5	101 ± 33	74 ± 10	368 ± 112	69 ± 6	95^{ab} ± 3	82 ± 6	715 ± 159
		5	94 ± 6	84 ± 7	330 ± 25	75 ± 10	96^{ab} ± 10	86 ± 10	681 ± 63
		10	78 ± 23	71 ± 13	298 ± 80	59 ± 9	84^{a} ± 11	75 ± 8	594 ± 131

Table 4
Concentration of kaempferol glycosides (ng g^{-1}) of pea seedlings after exposure to CAPP followed by 24 h adjustment. Different letters indicate significant ($p < 0.05$) differences between means.

Exposure to CAPP [min] as			Concentration of kaempferol glycosides [ng g^{-1}]					
Seeds	Sprouts	Seedlings	K-triglc	K-3-sin-triglc	K-3-cou-triglc	K-3-fer-triglc	K-3-cou-triglc (derivative)	Total KG
0	0	0	45 ± 3	34 ± 5	66 ± 3	40 ± 3	32 ± 5	218 ± 20
		2.5	41 ± 9	33 ± 6	58 ± 12	42 ± 22	32 ± 7	206 ± 40
		5	48 ± 20	43 ± 19	65 ± 28	52 ± 3	43 ± 18	251 ± 107
		10	45 ± 18	42 ± 18	62 ± 13	49 ± 15	42 ± 18	240 ± 81
	5	0	99 ± 60	90 ± 58	126 ± 66	96 ± 58	86 ± 57	497 ± 299
		2.5	77 ± 32	69 ± 29	107 ± 34	80 ± 29	68 ± 29	402 ± 152
		5	48 ± 12	42 ± 8	67 ± 25	50 ± 13	43 ± 8	249 ± 66
		10	44 ± 10	43 ± 8	59 ± 17	48 ± 11	42 ± 8	236 ± 53
5	0	0	82 ± 74	79 ± 77	100 ± 77	88 ± 79	79 ± 76	427 ± 383
		2.5	45 ± 20	45 ± 18	77 ± 24	53 ± 22	36 ± 20	256 ± 104
		5	42 ± 10	40 ± 8	60 ± 23	48 ± 14	39 ± 7	228 ± 63
		10	30 ± 14	24 ± 9	45 ± 19	31 ± 11	24 ± 9	154 ± 63
	5	0	46 ± 12	42 ± 13	75 ± 14	55 ± 7	40 ± 10	257 ± 57
		2.5	42 ± 6	34 ± 2	65 ± 3	46 ± 10	33 ± 2	221 ± 23
		5	45 ± 7	38 ± 8	65 ± 9	50 ± 9	38 ± 8	236 ± 42
		10	38 ± 8	33 ± 2	64 ± 15	45 ± 4	75 ± 3	213 ± 33

K: kaempferol; triglc: triglucoside; cou: *p*-coumaroyl; fer: feruloyl; sin: sinapoyl.

of ROS by constitutively high concentrations of quercetin glycosides, which provide a high antioxidant capacity (Zietz et al., 2010)

In general, the flavonol glycosides of pea seedlings tended to decrease dose-dependently with increased CAPP treatment at seed, sprout and seedlings stages, but independent of the pre-treatment. In contrast, UV-B treatments with five subsequent doses of moderate UV-B (0.5 kJ m^{-2} d^{-1} UV-B) resulted in higher concentrations of quercetin glycosides in kale at 15 °C (Neugart et al., 2014). Quercetin glycosides can function as antioxidants. Their content may decrease when high concentrations of ROS occur in the plant for a short time. In contrast, constantly high concentrations of ROS, resulting from the continuous exposure to high irradiation levels of sunlight (including UV light), led to increased concentrations of quercetin glycosides in linden leaves (Majer, Neugart, Krumbein, Schreiner, & Hideg, 2014). In the present experiment on pea, CAPP-treatment at three time points as seeds, sprouts and seedlings resulted in a decrease of quercetin glycosides. Consequently, we assume that the quercetin glycosides were used as antioxidants in the pea seeds, sprouts and seedlings due to a short time of exposure and high doses of UV-C.

4. Conclusion

The results of this study demonstrate that freshly germinated grain peas respond to exposure to CAPP produced from ambient air. In general, rates of seed germination increased with duration of plasma exposure. This was accompanied by reduced growth and increased dry matter content of evolved sprouts. The response of photosynthetic efficiency of seedlings and sprouts to CAPP exposure greatly depended on the growth stage of the peas at which the treatment had occurred and on the duration of CAPP exposure. Nevertheless, all treatments tested decreased the photosynthetic efficiency of seedlings. This is assumed to be related to the doses of ROS and UV radiation due to the plasma parameter selected. In addition, CAPP treatments modified the flavonol glycoside profile in pea seedlings, tending to dose-dependently decrease flavonoid contents due to high concentrations of UV-C for short exposure times, which did not act as a constant trigger for flavonoid biosynthesis. Subsequent low doses of plasma might possibly enhance the flavonoid glycosides in pea seedlings. Indeed, the plasma-induced effects on the flavonol glycoside profile may be a consequence of i) the impact on photosynthetic efficiency and on related signaling pathway(s) involved in the synthesis of plant secondary metabolites and ii) the protection against oxidative stress resulting from excessive strain by ROS and UV radiation from CAPP. Similarly, the chemical modification of the target molecules, which may be induced by plasma-immanent species, has to be considered. Further investigations should include the analysis of plasma-initiated reactions and the effect of subsequent low doses of CAPP to gain deeper knowledge in plasma-dose–response-relationships for the production of a health-promoting functional food. Hence, this study evidences the potential to implement CAPP technology for a targeted modification of valuable secondary plant metabolites during post-harvest chain of agricultural produce.

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4.1.2 Characterization of individual proteins in pea protein isolates and air classified samples

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Characterization of individual proteins in pea protein isolates and air classified samples

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ABSTRACT

Generally, pea proteins are extracted at comparatively acidic or basic pH values to provide a basis for protein isolate production. Such processing steps result in partial denaturation of the proteins rendering them in most cases insoluble at food processing pH conditions and limiting their application in food products. Here, the comparison of the solubility properties of pea proteins in protein enriched fractions deriving from air classification is reported. Protein content, solubility, and physicochemical parameters of different fractions of the pea (*Pisum sativum*) variety 'Salamanca' were investigated as a function of pH using SDS-PAGE and surface hydrophobicity. Whole pea flour (20% protein), air classified, protein-enriched pea flour (48% protein), pea flour made from hulls (2.8% protein), and pea protein isolate (81% protein) served as test materials. Fractionation and pH value affected the composition and surface hydrophobicity of the proteins as well as the content of trypsin inhibitors. All samples showed a high buffering capacity in the range of pH 4 to 10. The direct comparison documents the comparatively better protein quality of the air classified, protein enriched pea fraction. The solubility of the pea protein isolate can be improved by using selected additives, giving new possibilities for plant protein application. Relevant technofunctional properties were determined and compared with two commercially available pea-based products (whole pea flour and an isolate). Water binding capacity was highest for the commercially available pea flour followed by the pea hull flour. Fat binding capacity remained more or less unchanged.

1. Introduction

The steady increase in the world population in recent years has caused the local governments to develop new strategies for the coverage of the required protein consumption. As a consequence, governments are searching for alternative, sustainable local products for being integrated in long-term protein utilization strategies. In Germany, as well as in other European countries, a revival of domestic grain legumes has been encouraged. So far, the largest share of imports is accounted by soybeans (Schaack, 2011). With a protein content of approximately 40%, soybean constitutes a valuable protein source for food as well as feed (Mujoo, Trinh, & Ng, 2003; Vollmann, Fritz, Wagentristl, & Ruckenbauer, 2000; Wolf, 1970). The well-developed food processing options for soybean and soybean proteins have been the main competition aspects against the production of domestic (grain) legumes in (Northern) Europe.

Legumes such as beans, lentils, chickpeas, and peas (*Pisum sativum*) sluggishly return into the research focus (Barac et al., 2010; Costa, Queiroz-Monici, Reis, & de Oliveira, 2006; Wang, Hatcher, Warkentin, & Toews, 2010). This revival makes the utilization of components from legumes as innovative ingredients for the food industry interesting (Schaack, 2011). Re-emerging legumes such as beans and peas being also rich in starch, fibre, vitamins, and minerals have received more attention as alternative sources for providing protein-rich intermediates (Costa et al., 2006; Tharanathan & Mahadevamma, 2003). Exemplarily, depending on genotype and ecophysiological factors, protein content of peas ranges from 23.3% to 31.7% among different pea varieties (Barac et al., 2010; Wang, Hatcher, & Gawalko, 2008). These proteins comprise mainly of globulin storage proteins, representing 65–80% of the total-protein (Schroeder, 1982). In comparison to soybeans, pea proteins have higher levels of lysine, but less sulphur containing amino acids (Gruber, Becker, & Hofmann, 2005; Leterme, Monmart, & Baudart, 1990). The total pea proteins can be roughly divided into two major groups, the so-called 'albumins' and 'globulins' (Fukushima, 1991; Lakemond, de Jongh, Hessing, Gruppen, & Voragen, 2000; Sessa, 2004). The major proteins found in pea seeds belong to the storage proteins (ca. 90%). The globular proteins mostly exist in native conditions as oligomeric structures belonging to 7S and 11S (or 15S) fractions (Fukushima, 1991). The major pea storage proteins are often referred to as legumin (11S), vicilin (7S), and convicilin composing the globulin fraction. The pea 11S storage proteins of legumes are built up of subunits determined by multigene families. They form hexameric, quaternary structures (molecular weight ca. 320 to 380 kDa). Their

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association–dissociation and their surface structure are the most important factors for understanding their functionality (Ali et al., 2012; Barac et al., 2010; Fukushima, 1991). The legume 7S fractions have molecular weights of 150–200 kDa and constitute a trimer molecule composed of α and β sub-fractions frequently termed vicilin proteins (Fukushima, 1991; Shewry, Napier, & Tatham, 1995). They lack cysteine residues and hence cannot form disulphide bonds, although reversible aggregation into hexamers, depending on the ionic strength is possible (Shewry et al., 1995). The differences in content, composition and structure between vicilin and legumin are exhibited in both nutritional and technofunctional properties as recently summarized in Barac et al. (2010). Finally, a further major globulin protein was reported as convicilin (71–75 kDa), and differs from vicilin in that it does have sulphur containing amino acids (Croy, Gatehouse, Tyler, & Boulter, 1980). The characteristic amino acid content also differs for legumin, vicilin, and convicilin in sulphur containing amino acids and has been compared by Croy et al. (1980).

Compared to traditional flours made from cereals, pea flour does not contain any gluten and could be a useful contribution for the production of gluten-free foods (Han, Janz, & Gerlat, 2010; Mariotti, Lucisano, Pagani, & Ng, 2009). Moreover, legumes and innovative products developed thereof can contribute to secure sufficient, sustainable foods for vegetarians and vegans. In contrast to soybeans, the other legumes do not or hardly contain phytoestrogens which are discussed controversially in terms of hormonal activity and the potential of triggering certain types of cancer (Allred et al., 2004) Peas can contain a number of putative anti-nutritive compounds (e.g. protease inhibitors, lectins, and saponins). However, these are also discussed quite controversially these days: Exemplarily, compared to many other saponins, saponins from peas are not haemolytic (Reim & Rohn, 2015). Even protease-inhibitors seem to be able to provide protection to some extent against degenerative diseases such as cancer (Park, Jeong, & Lumen, 2007).

Nonetheless, there is a need to develop processing steps which will minimize such components, as long as their positive aspects have not been proven comprehensively. Preliminary experiments showed differences in the content and trypsin activity of the various pea varieties investigated, establishing that summer cultivated pea varieties have a lowered activity. In this context, one aim of the present study was to characterize the proteins from the summer variety 'Salamanca' under varying extraction conditions and to investigate how the composition and properties of recovered concentrates and isolates are affected. The pea variety 'Salamanca' is widely cultivated in Germany because of bringing high yields, providing a high protein content and ripening comparatively early under the local climatic conditions. A further goal of the present study was to compare several technological processing steps resulting in protein-rich intermediates with regard to their solubility behaviour and the content of trypsin inhibitors to initiate new recommendations for the their production.

For these purposes, the intermediate products pea flour (PF), protein-rich pea flour (PPF), pea protein isolate (PPI), and pea flour from outer hulls (*pea testa flour* — PTF) of pea seeds, which can be also used for the industrial processing or directly for the consumers, were characterized. The effect of pH on solubility, trypsin inhibitor activity and selected functional properties of pea protein concentrates and isolates was additionally investigated to predict their application in food processing.

2. Materials and methods

2.1. Materials

Yellow pea seeds (*Pisum sativum* 'Salamanca'; 'Gregor', and 'James') were provided by Norddeutsche Pflanzenzucht, Hans Georg Lembke GmbH (Hohenlieth, Germany) and processed by Institut für Getreideverarbeitung GmbH (Nuthetal, Germany). The preliminary experiments were performed with the above-mentioned three varieties in comparison to commercially available soy flour (Sigma Aldrich Chemie GmbH Steinheim, Germany) to determine the suitability of 'Salamanca' for further characterization based on their whole seed protein and trypsin inhibitor contents. Hulling of whole seeds was performed with a shelling machine (F.H. Schule Mühlenbau GmbH, Reinbek, Germany) and ground using a grinding mill (Rekord A, Mez, Prag, Czech Republic). The maximum particle size of pea flour was allowed to be 500 μm. Besides the flour of the pea endosperms, the finely ground outer layers (hulls), protein enriched pea flour and pea protein isolate served as basis for this study. Pea flour from the outer layers was prepared using a centrifugal mill (Ultra Centrifugal Mill ZM 200, Retsch, Haan, Germany). For preparing the protein enriched pea flours, hulled seeds were separated into different fractions with a ConduxCSM 80 classifier mill (Erich NetzschGmbH & Co. Holding KG, Selb, Germany). Air classification is a milling technique that allows the fractionation of grains/seeds into high starch and high protein flours. During the air classification, the light fine fraction (protein) is separated from the heavy coarse fraction (starch), where the flour is subsequently classified in a spiral air stream to separate the starch from the protein (Boye, Zare, & Pletch, 2010). The process can be repeated several times to improve the separation efficiency.

Total nitrogen content of all pea flour (fractions) was estimated using the Kjedahl method with a conversion factor of 6.25. In comparison to these 'Salamanca' samples, two commercial products (pea flour — cPF, Caremoli Deutschland GmbH, Stuttgart, Germany; pea protein isolate — cPI; Emslandstärke GmbH, Emlichheim, Germany) were also analysed.

Chemicals for the analyses were of reagent grade.

2.2. Preparation of pea protein isolate

PPF was dispersed in distilled water at a ratio of 1:8, i.e. 50 g flour in 400 g water. The pH was adjusted to pH 1.5 using concentrated hydrochloric acid. For extracting the soluble pea proteins, the dispersion was stirred at room temperature for 30 min using a magnetic stirrer. Dispersion was centrifuged for 60 min at 4000 ×*g*, and 4 °C (Megafuge 2.0 R, Heraeus Sepatech GmbH Düsseldorf, Germany). The clear supernatant was collected, following pH adjustment to 4.5 using 1 M sodium hydroxide solution for precipitating the proteins. The precipitate, supported by a centrifugation step (60 min at 4000 ×*g*, and 4 °C) was frozen and lyophilized (Christ Alpha 1–4 Gefriertrocknungsanlage, Christ Gefriertrocknungsanlagen Osterode, Germany). Samples were stored at −20 °C until analysis.

2.3. Physicochemical and structural characterization

2.3.1. Protein content and solubility

The solubility profile of the proteins from the samples PF, PPF, PPI, and PTF was determined under varying the pH values (distilled water from 1 to 12). For this purpose, the protein content of the solutions was determined according to the Bradford method. Samples were dissolved/extracted by adding 25 mL of distilled water (adjusted to the corresponding pH value) to 500 mg of PF, PPF, PTF, and PPI. All extractions were conducted in triplicate. After stirring for 30 min at room temperature, the suspensions were centrifuged at 6000 ×*g* for 30 min at 4 °C. Aliquots of the supernatant were filled in tubes and analysed.

2.3.2. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE according to the method of Laemmli (1970) was used for determining the molecular weight distribution. The samples (n = 3) were pooled and mixed in a ratio of 1:1 with sample buffer (0.05 M Tris–HCl buffer at pH 6.8 containing 4 g of sodium dodecyl sulphate, 12 g of glycerol, 5 g of 2-mercaptoethanol, and 0.01 g of Coomassie Brilliant Blue R 250). Samples were prepared by denaturing the proteins at 95 °C for 3 min prior to analysis. The gels were prepared with the

vertical electrophoresis equipment from biostep GmbH (Jahnsdorf, Germany). Low molecular weight calibration kit for SDS-PAGE was used (Amersham LMW Calibration Kit, Pharmacia, GE Healthcare, Buckinghamshire, UK) and 10 μL of the samples were separated in 14% T gels. The band intensity was estimated after staining with Coomassie Brilliant blue and corresponding de-staining protocols. Quantification was done using Quantity One 1-D Analysis Software, version 4.5.2 (Bio-Rad, Universal Hood II, Bio-Rad Laboratories, Segrate, Milan, Italy).

2.3.3. Surface hydrophobicity

The surface hydrophobicity of the samples was determined using the hydrophobic fluorescence probe, 1-anilino-8-napthalensulphonate (ANS) according to the modified method of Schmitt, Bovay, Rouvet, Shojaei-Rami, and Kolodziejczyk (2007). ANS is an environmentally sensitive fluorophore used to observe structural changes in the surface of proteins. A stock solution of ANS (0.001 M in a 0.01 M phosphate buffer, pH 7; Sigma-Aldrich Chemie GmbH, Munich, Germany) was prepared and diluted to concentrations between 60 and 300 μM. In order to ensure a protein concentration of the samples ranging from 0.125 to 0.5 mg/mL, all protein extracts were diluted adequately (1:5) in 0.05 M phosphate buffer (pH 7). Fluorescence signals were measured using a plate reader (Fluostar OPTIMA, BMG Labtech GmbH, Ortenberg, Germany). Samples were filled into a 96-well plate. The excitation wavelength was set to 370 nm and the emission wavelength was set to 470 nm. The maximum fluorescence intensity Fmax was considered to correspond to the saturation of all available hydrophobic sites at the surface of the soluble pea proteins by ANS. The required ANS concentration to obtain a fluorescence intensity of Fmax/2, was defined as the apparent dissociation constant kD. The ratio Fmax/kD was calculated and defined as the protein surface hydrophobicity index (SHI).

2.3.4. Influence of arginine and sodium carbonate on the solubility of PPI

The best solubility of the PPI is known to be at extreme pH values (2 or 12) and therefore the conditions for an effective use of PPI in the food industry must be optimized. So the influence of the solubility of the PPI through the addition of food relevant substances (arginine, sodium carbonate) was analysed. A stock solution of arginine and sodium carbonate (5%) in distilled water was prepared and diluted in different concentrations (4%, 3%, 2%, 1%, 0.5%, 0.1%, and 0.05%). Then PPI (0.2%) was dissolved in distilled water, or in the different arginine or sodium carbonate solutions. As controls, 1% SDS and 8 M urea were used. The solutions were treated with ultrasound for 1 min, stirred for 30 min, and centrifuged (5 min, 10,400 ×*g*, 4 °C). After that, the extinctions of the supernatants were measured at 280 nm, because the amino acids, tryptophan and tyrosine have strong absorption bands at this wavelength. Additionally, the pH values of the different samples were analysed. In another experiment we modified the concentration of PPI (0.2%, 0.5%, 1%; 1.5%, 2%) in an arginine solution (0.1%).

2.3.5. Trypsin inhibitor activity

Trypsin inhibitor activity (TIA) of the samples was determined according to the method of Kakade, Rackis, Mcghee, and Puski (1974) with minor modifications. TIA was measured at 410 nm using N_{α}-Benzoyl-D, L-Arginin-p-nitroanilin (BAPNA, AppliChem GmbH, Darmstadt, Germany) as enzyme substrate and measured with a platereader (Fluostar OPTIMA, BMG Labtech GmbH, Ortenberg, Germany). Sample controls for each sample and reagent controls, containing buffer instead of sample were determined (TIA = 100%). TIA has been defined as trypsin inhibition units (TIUs), where one trypsin unit (TU) corresponds to an increase of 0.01 absorbance units at 410 nm.

2.3.6. Water binding capacity

The method by Smith and Circle (1978), modified by Quinn and Paton (1979) was used for determining the water binding capacity of the pea flour fractions. The required amount of water was calculated by the method of Smith and Circle (1978), modified by Schwenke et al. (1981). For this purpose 0.5 g of the corresponding pea flour fractions was weighted into a 15 mL centrifugation tube and stirred (60 s) with 2.5 mL tap water using a propeller stirrer and an overhead agitator (IKA, New Jersey, USA). After 20 min centrifugating at 3900 ×*g*, the supernatant was decanted and the tube was put upside-down on a filter paper for 60 min and finally weighed. Water binding capacity was calculated as:

$$((F-I)/IDM) \times 100 \quad (1)$$

whereby, I is the initial weight of the sample, F is the final weight of the sample, and IDM is the initial weight of the sample based on dry mass determined.

2.3.7. Fat binding capacity

For the determination of the fat binding capacity, the method of Schwenke et al. (1981) was used. The respective pea flour fraction (0.5 g) was weighed into a 15 mL centrifugation tube and stirred with 5 mL of commercial rape seed oil using a propeller stirrer and an overhead agitator (IKA, New Jersey, USA) two times for 60 s with a five-min intermission between the two stirring steps. After 20 min centrifugating at 3900 ×*g*, the supernatant was decanted and the tube was put upside-down on a filter paper for 60 min and finally weighed. Fat binding capacity was similarly calculated as described in Eq. (1).

2.3.8. Statistical analysis

All experiments were conducted at least three times. The number of parallel samples in the experiments was three or more. All data are expressed as means ± standard deviations.

3. Results and discussion

3.1. Preliminary observations

The typical and widely accepted pea varieties in Germany 'Salamanca'; 'Gregor,' and 'James' were initially investigated with regard to their protein content and trypsin inhibitor potential. The results were compared with commercially available soy flour and are presented in Fig. 1. The protein contents of the three flours range from 21–23% and most of these are well extracted (17–23% protein) with 0.01 M NaOH as determined by the Bradford method (Fig. 1). while investigating the trypsin inhibitor activity under alkaline conditions (Fig. 1, Bradford values). In this context, pea protein content from three cultivars and three different experimental lines grown in Serbia also ranged from 22–32%, whereas those that have been extracted under milder alkaline conditions (pH 8) were in the range of 9–12% (Barac et al., 2010).

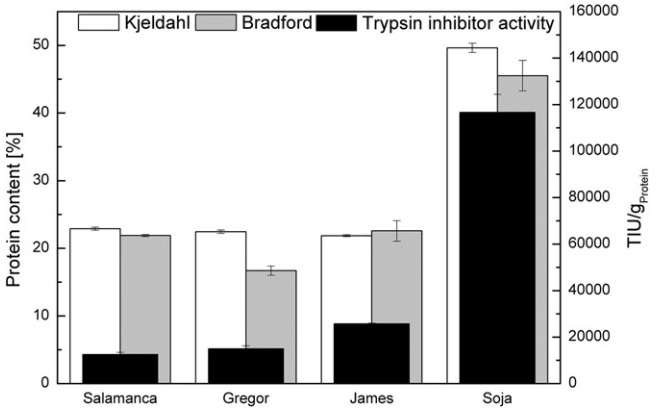

Fig. 1. Protein content and trypsin inhibitor activity of three different pea varieties (whole seeds of 'Salamanca', 'Gregor' and 'James') in comparison to soy flour. (TIU: trypsin inhibitor units); n = 3.

The content of trypsin inhibitor in pea seeds was in the range of 12,000–26,000 TIU/g protein as compared to relatively high protein content (50%) and corresponding 117,000 TIU/g protein for soy flour. The content of trypsin inhibitor in soy has been reported to be below 0.5% (Pusztai, Watt, & Stewart, 1991) and the activity varies greatly, but being significantly higher than in peas. According to the literature, seed samples of 17 field pea cultivars showed values ranging from 2.2–7.7 TIU/mg dry matter (Chang, Liu, & Tsai, 2014). The values are surely depending on a series of factors such as climate, plant nutrition, and further cultivation conditions as well as genotype (Alikhan & Youngs, 1973; Chang et al., 2014). In the present study, the lowest trypsin inhibitor activity (approx. 12,600 TIU/g protein) was found in the variety 'Salamanca' and therefore further experiments were conducted with this variety.

The variety 'Salamanca' was accepted for cultivation in Germany in December 2009. Despite its relatively long plant height, 'Salamanca' is characterized by high stability during plant growth accompanied with a constant high yield performance. This variety can also be used as an appropriate plant protein source for the food as well as feed sector. The characterization of pea flour (PF), protein-rich pea flour (PPF), pea testa flour (PTF), and pea protein isolate (PPI) was performed as a function of pH value. In contrast, the technofunctional properties of the pea flour fractions were determined depending on the milling process. Fig. 2 gives an overview of the preparation and the corresponding analyses.

The crude protein contents of PF, PPF, PTF, and PPI are presented in Table 1. In comparison to these 'Salamanca' samples, two commercial products (pea flour, Caremoli Deutschland GmbH, Stuttgart, Germany; pea protein isolate, Emslandstärke GmbH, Emlichheim, Germany) were also analysed. The protein content determined for both PF and PPI are comparable with the protein content of the commercial products and results obtained in other studies (Barac et al., 2010), while the PPI in the present study was produced in contrast by acidic extraction with PPF as initial raw material. For example, Boye, Aksay, et al. (2010) found 21.1% protein in pea flour from yellow peas and 81.7% in concentrates prepared by alkaline extraction followed by isoelectric precipitation. The content may vary depending upon cultivar. Up to 34% crude protein content was reported with a total protein content ranging from 84–89% in the corresponding isolates (Barac et al., 2010; Park, Kim, & Baik, 2010). Recent investigations encompassing 18 different pea cultivars and 42 different breeding lines showed crude protein content up to 26% (unpublished data). Furthermore, a variation in protein content was described depending on genotype and environmental influences (Alikhan & Youngs, 1973; Barac et al., 2010; Wang et al., 2008; Wang et al., 2010), therefore in this study the same batch of peas was used for all analysis procedures. The protein content of the pea flour was more than doubled when using air classification, whereas the yield of protein recovery in the corresponding PPI prepared from PPF was limited to 81.2% (Table 1). In contrast, the protein yield of PPI can be modulated to be higher than 90% Boye, Zare, et al., 2010.

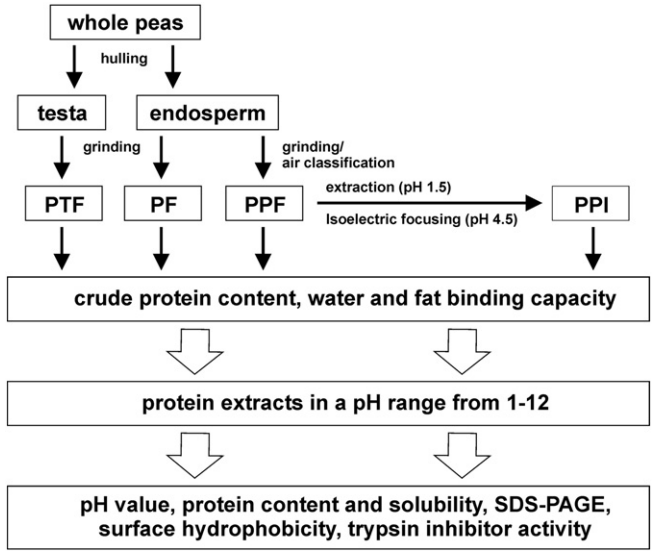

Fig. 2. Schematic fractionation of the pea seeds and production of the pea protein isolate. (PF: pea flour, PPF: protein-rich pea flour, PTF: pea testa flour, and PPI: pea protein isolate).

Table 1
Crude protein content of different pea flour fractions of 'Salamanca' and two commercial pea products. Data represent the mean values (± standard deviation) (n = 3).

Pea flour fraction	Crude protein content [%]
Pea flour — PF	20.04 (±0.11)
Protein-rich pea flour — PPF	48.26 (±0.21)
Pea hull flour — PTF	2.81 (±0.01)
Pea protein isolate — PPI	81.19 (±1.77)
Commercial pea flour — cPF	22.20 (±0.34)
Commercial pea protein isolate — cPI	80.23 (±1.25)

3.2. Solubility as related to the extracted proteins and their composition

Protein solubility is one of the most important physicochemical properties, because it affects the functional behaviour of proteins such as emulsification and foaming (Cheftel, Cloarec, Moretti, Rafelson, & Jayle, 1960) and thus the application of proteins in food products. Protein solubility is largely influenced by the extraction conditions, e.g. pH value, temperature, and ionic strength (Khalid, Babiker, & EL Tinay, 2003; Lawal, 2004; Ragab, Babiker, & Eltinay, 2004), The protein contents extracted have been variable, probably due to differences in such processing conditions (Boye, Zare, et al., 2010). To simplify this particular processing step, water was adjusted to different pH values and added to the samples and the solubility determined. The pH was not re-adjusted and the proteins were able to enfold their own buffering potential. In Fig. 3, the protein solubility is depicted as na function of the pH value for PF, PPF, PTF, and PPI. The results show both the protein yield (Kjedahl and Bradford) and composition as analysed by SDS-PAGE. A direct comparison of PF, air-classified samples (PPF, PTF), and the PPI produced from PF is thus possible.

At a first glance the protein solubility profile of pea flour (Sosulski & McCurdy, 1987) and pea protein isolates (Boye, Aksay, et al., 2010; Taherian et al., 2011) differs from that of *Pisum sativum* 'Salamanca' proteins investigated in the present work. In the studies cited, the lowest solubility was reported to be at pH values between 4 and 6. But in contrast to Boye, Aksay, et al. (2010), the pH of the extraction solution was adjusted from pH 1 to 12 with either 1 M HCl or 1 M NaOH and then mixed with PF, PPF, PTF, and PPI. After 30 min extraction time the pH of the protein extracts were determined (Table 2). Table 2 and Fig. 3 reveal that the protein solubility is also lowest at pH 4–6 in the protein extract from PF and PPF, which corresponds to a pH value of 2 or 3 respectively in Fig. 3. All of the recovered protein extracts showed high buffer capacities at pH values from 4 to 10 of the solvent. Protein extracts recovered from PF and PPF showed values in the same range (pH 6.6–6.8; Table 2). Therefore, under these conditions there is a re-adjustment of the initial solvent pH-values by the buffering capacity of the extracted proteins. About 30–60% can be recovered for 'Salamanca' at this finally re-adjusted pH 6–7, as also reported for both PF and PPI from other pea varieties (Boye, Aksay, et al., 2010; Sosulski & McCurdy, 1987; Taherian et al., 2011). The highest solubility was at 11–12 for all fractions due to the effect of pH being willingly adjusted. For PPI, the highest protein solubility was found to be at pH 12, too. But the protein yield at pH 2 was slightly higher (Fig. 3). An interesting observation is the fact that the protein solubility in the range of pH 4–11 is very low (approx. 10%) in comparison to PF and PPF. This could be a result of the acidic extraction and isoelectric precipitation which in turn influences the protein conformation and composition. A lower solubility of a commercial PPI at pH 2–9 was also documented (Taherian

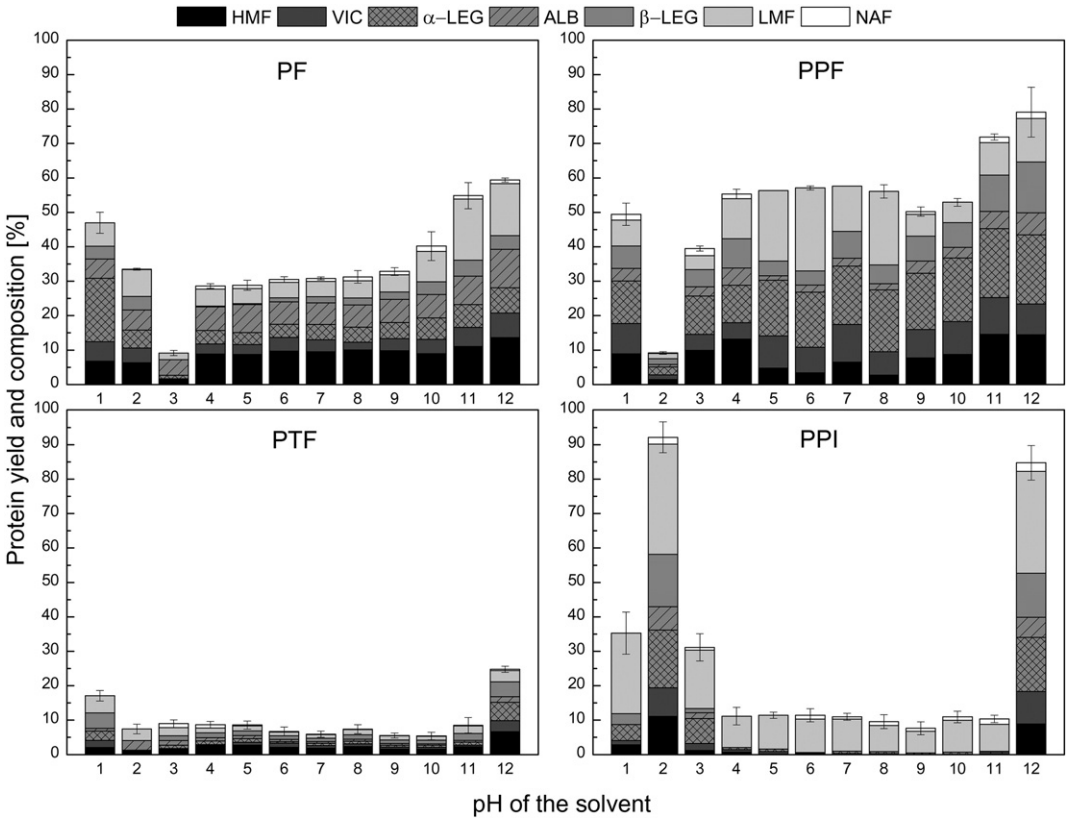

Fig. 3. Composition of the pea protein fractions (PF: pea flour, PPF: protein-rich pea flour, PTF: pea testa flour, and PPI: pea protein isolate) depending on the pH value of the extraction solution. (HMF: high molecular fraction, VIC: vicilin, α-LEG: α-legumin, ALB: albumin, β-LEG: β-Legumin, LMF: low molecular fraction, NAF: none allocated fraction); n = 3.

et al., 2011) and the authors explained these observation as being based on an increase in exposed hydrophobic residues, leading to an increased hydrophobic interaction between proteins and/or peptides in the acidic pH region as also stated elsewhere (Tsumura et al., 2005). In case the pH value can be maintained or a buffer has been applied while extracting, 'Salamanca' PPI was also found to behave as reported elsewhere, with the lowest solubility to be found between pH 4–6 (data not shown). The testa of 'Salamanca' has generally low protein content (Table 1) and the extraction at pH 12 led only to a protein proportion of 30% (Fig. 3). The major proteins in this fraction (PTF) may include largely membrane-bound or highly hydrophobic proteins, which are not easily extracted under these conditions.

In order to understand the variability of the protein composition during the extraction/solubilization process, it is necessary to allocate the major components as analysed by SDS-Page for PF (supporting information). This approach allows the assessment of the protein fractions soluble under different conditions as discussed in the following.

Based on this allocation, the composition of individual fraction distribution in the extracted proteins for the four pea samples is also given in Fig. 3. In case PF, a better solubility of the aggregated proteins (HMF fraction) and low molecular components (LMF) was observed, except at pH 3. The solubility also increased with increasing pH of the extraction solution. The storage proteins (LEG and VIC) are more efficiently extracted at the extreme pH conditions (pH 1, 11, or 12). An interesting observation is the relatively high content of the α-chains of legumin at pH 1. The solubility behaviour is changed while preparing the PPF. In this case the storage proteins are more efficiently concentrated and accordingly dominate the extracted fractions (Fig. 3). As observable from Fig. 3, VIC fraction is also involved in the insoluble part of the protein, esp. in case of PF and PPI, and as it does not have any sulphur containing amino acids esp. cysteine, the role of crosslinking via disulphide bridges may play only a secondary role. The PPI sample preparation induces a change in the structural properties resulting in their insolubility over the pH range 3–11. Only at extreme values pH 2 and 12 is a reliable solubility of all the protein fractions given. The PTF fraction that should actually represent the majority of albumins, also contains the other classified fractions.

These results indicate that it might be useful to prepare a protein enriched fraction such as PPF rather than a protein isolate (PPI), as a better solubility of the storage proteins is guaranteed over a broad pH range. A further opportunity is given by using different modes of preparations and thereby influencing the composition and correspondingly the functional and nutritional properties of the protein enriched intermediates. Under defined conditions, it is possible to obtain PPI with better solubility profiles e.g. when using ultrafiltration techniques (Boye, Zare, et al., 2010; Tsumura et al., 2005). The commercial PPIs, traditionally prepared, generally lack good solubility profiles. Selective hydrolysis may also improve these issues and could be an alternative

Table 2
pH values of the protein extracts in distilled water (pH 1–12) after extraction time of 30 min. Data represent the mean values (± standard deviation) (n = 3).

pH of the solvent	pH of the protein extract [−]			
	Pea flour	Protein-rich pea flour	Pea hull flour	Pea protein isolate
1	0.91 (±0.01)	1.28 (±0.01)	1.33 (±0.03)	0.96 (±0.03)
2	2.93 (±0.09)	5.04 (±0.01)	4.051 (±0.01)	2.17 (±0.01)
3	6.02 (±0.02)	6.37 (±0.01)	5.37 (±0.03)	3.63 (±0.03)
4	6.70 (±0.02)	6.58 (±0.01)	5.66 (±0.02)	3.96 (±0.02)
5	6.84 (±0.04)	6.58 (±0.03)	5.66 (±0.01)	3.97 (±0.03)
6	6.82 (±0.08)	6.58 (±0.01)	5.71 (±0.02)	3.96 (±0.01)
7	6.78 (±0.02)	6.58 (±0.02)	5.68 (±0.01)	3.98 (±0.01)
8	6.84 (±0.03)	6.59 (±0.02)	5.65 (±0.03)	4.04 (±0.06)
9	6.87 (±0.04)	6.59 (±0.01)	5.72 (±0.04)	4.01 (±0.02)
10	6.80 (±0.02)	6.61 (±0.02)	5.90 (±0.01)	4.16 (±0.01)
11	8.39 (±0.08)	6.99 (±0.01)	7.38 (±0.03)	6.12 (±0.01)
12	11.57 (±0.01)	9.93 (±0.01)	10.68 (±0.03)	11.32 (±0.01)

approach (Tsumura et al., 2005). However, adding pepsin during protein extraction at acidic pH values did not provide an adequate solution (data not shown). It appears that the major pea protein components once freed from the insulating starch-rich matrix, tend to interact strongly with one another, resulting in aggregation with unfavorable surface properties and thereby promoting increased ionic or hydrophobic protein–protein interactions.

3.3. Solubility as related to hydrophobicity of the extracted proteins

A good correlation between insolubility and hydrophobicity has also been reported for different proteins e. g. milk and soy proteins (Hayakawa & Nakai, 1985). Therefore, in the next step of the present study, 1-anilino-8-napthalensulphonate (ANS), a much-utilized fluorescent 'hydrophobic probe' known to bind to hydrophobic pockets on the surface of the proteins, was used for investigating the non-polar character of proteins extracted. By plotting the fluorescence intensity versus the protein concentration, an estimation of the fluorescence increase per unit protein concentration (surface hydrophobicity index, SHI) can be obtained by using the slope. SHI of all protein samples are shown in Fig. 4. Among the four fractions, PPI has highest SHI at pH values 4–5 (172 or 210 mV/μM) and the lowest at solvent pH 2 (26.6 mV/μM) contrary to the others, which have a SHI maximum at pH 2. PTF has the highest SHI over the entire pH range, but the lowest protein content. The highest and lowest SHI in PTF was obtained at pH 1 and 2 in the solvent (424 or 436 mV/μM), and at pH 11 and 12 in the solvent (212 or 221 mV/μM), respectively. As expected, SHI of PF and PPF is constant over the range from pH 4 to 10 due to comparable pH and protein content. But it was also expected that SHI of PTF and PPI showing the same behaviour in the pH range from 4 to 10, but this was not the observed in this case. These results show that pH greatly influenced SHI, but there is no direct correlation between pH and surface hydrophobicity in the pea protein fractions, except for the hull fraction (PTF) which may include largely membrane-bound or highly hydrophobic proteins. These can be concentrated and may provide alternative utilization options. Consequently, ionic interactions may also be relevant in maintaining the protein–protein interactions resulting in limited solubility of the newly formed aggregates during the extraction process.

3.4. Improvement of the solubility by additives

In order to improve the solubility of PPI, the protein–protein interactions need to be kept at a minimum. Additives such as urea and SDS are known to denature and insulate the protein molecules providing a higher solubility (Fig. 5A). L-Arginine is classified as a semi- or conditionally indispensible amino acid. It is also the immediate precursor of urea being structurally similar in that the distal end of it is capped by a complex guanidinium group. The addition of arginine improves the solubility of PPI as shown in Fig. 5A, also accompanied by a corresponding increase of the solvent pH value. Further, it was observed that at least half of the amount of arginine to that of the PPI is sufficient for acquiring a better solubility, while decreasing the ratio of arginine to PPI, a decrease of the protein solubility is observed (Fig. 5B). These observations also confirm that the role of crosslinking via disulphide bridges may only play a secondary role in the insolubilization of these protein fractions. The addition of sodium carbonate to increase the pH has only a slight effect for improving the solubility (Fig. 5A). Similar experiments with the anti-flocculant lecithin did not increase the solubility (data not shown). L-Arginine may be safely used when added to foods. It is widely used as nutrition supplement. It is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA). In this context, a highlighting application is the clinically proven benefit of rapid and lasting relief of dentin when applying arginine in combination with calcium carbonate (Petrou et al., 2009). So, arginine may also provide a useful tool in solubilizing PPI during food processing.

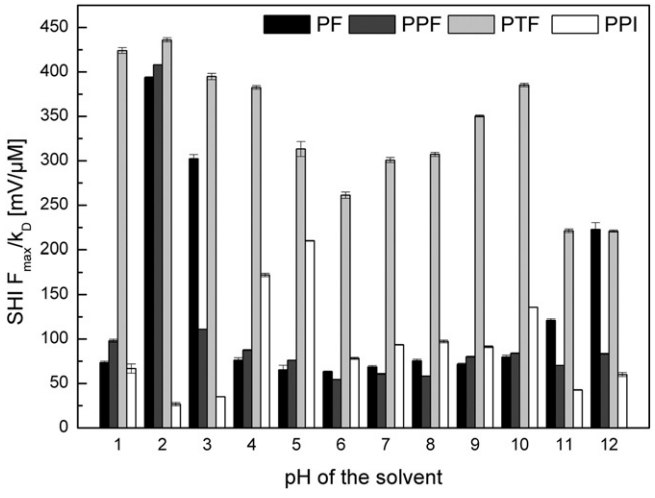

Fig. 4. Surface hydrophobicity index SHI [mV/μM] of the pea flour fractions. (PF: pea flour, PPF: protein-rich pea flour. PTF: pea testa flour, and PPI: pea protein isolate); n = 3.

3.5. Solubility as related to content of anti-nutritive components

From nutritional point of view, it is necessary to evaluate the pea samples thus prepared by air classification with regard to their content of anti-nutritive components. As a marker for this quality feature, the trypsin inhibitor activity (TIA) was exemplarily determined. Trypsin inhibitors (TI), belong to the albumins, as already described above. In selected legumes such as common bean (*Phaseolus vulgaris*), lentil (*Lens culinaris*), and pea (*Pisum sativum*), the protease inhibitors have been characterized and classified as Bowman-Birk inhibitors (Ferrasson, Quillien, & Gueguen, 1995; Guillamon et al., 2008; Lajolo & Genovese, 2002). The function of TI in physiological and functional processes in plants is still not yet clarified. It is assumed that TI may improve sulphur storage and plays a role in the regulation of endogenous proteinases during germination and for protecting against insects and microbes (Ryan, 1973). The presence of TI limits the utilization of raw pea seeds in animal nutrition, due to their associated decreased protein digestibility and poor growth performance in animals (Jondreville, Grosjean, Buron, Peyronnet, & Beneytout, 1992; Leterme, Beckers, & Thewis, 1990). High trypsin inhibitor activity was found in PTF over the entire pH range (Fig. 6). Dehulling of pea seeds and air classification of PF resulted in a reduction of TIA (Fig. 6). PPF has a very low TIA (1041–14,048 TIU/g protein) for all pH values tested. The most interesting pH values of the PPI are pH 2 and 12, as at these values the corresponding protein content is very high (Fig. 3). The low TIA value at pH 2 (1524 TIU/g protein) and 12 (1025 TIU/g protein) of the PPI documents, that the protein extraction and the consequent isoelectric precipitation at pH 4.5 of the proteins extracted from PF also has a positive effect. As expected, TIA of each fraction (PF, PPF, PTF, PPI) in the buffering region (pH 4–10) is approximately in the same range (Fig. 6). For PF, comparable values for TIU were obtained to those reported in the literature for four pea varieties ranging from 4000–84,000 TIU/g protein (Guillamon et al., 2008). With regard to the present results, the method of air classification provides the best strategy for the preparation of protein-enriched food intermediates with low trypsin inhibitor content.

3.6. Effect of air classification on other technofunctional properties

When addressing the potential application of such protein enriched fractions, it is also necessary to compare their further technofunctional properties. Significantly different emulsifying, foaming properties, as well as water and oil holding capacity between flour prepared from different pea varieties have been discussed (Barac et al., 2010). The water binding capacity (WBC)

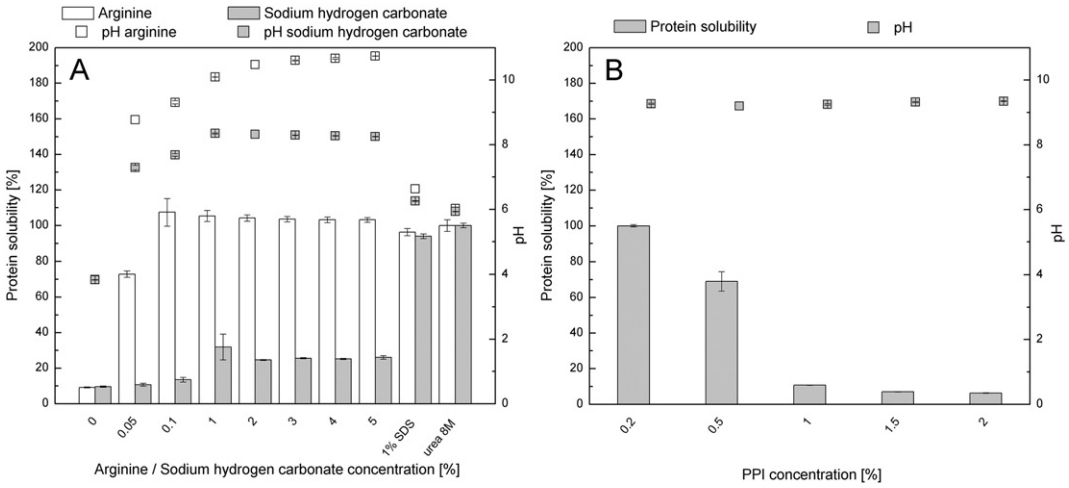

Fig. 5. A and B. Influence arginine on the solubility of PPI. (A) Influence of different concentrations of arginine and sodium carbonate; (B) Influence of different concentrations of PPI in a 0.1% arginine solution n = 3; PPI: pea protein isolate.

and fat binding capacity (FAC) of the fractions of 'Salamanca' and the two commercial products are presented in Fig. 7. Among the fractions assessed, PF has the lowest WBC and FAC. The commercially available cPF has the highest WBC and FAC. This result was not expected, because both are flours with similar protein content (Table 1). WBC and FAC are not only depending on protein content, because the content of PPI and cPI is much higher and the selected properties are lower. The highest WBC between the fractions of 'Salamanca' was shown for PTF followed by PPI with a three times higher capacity than PF. Thus, the WBC will be affected by the selected processing steps, but there is only a very slight effect on the FAC. The comparison of the PF with the commercially available cPF shows large differences in WBC/FAC, indicating further potentials in technological processing of pea flours. Both, PPI and commercially available products (cPPI) have similar binding capacities. Interestingly, the PTF fraction, although having high surface hydrophobicity, has a rather low FAC but a higher WBC. These results indicate that the technofunctional properties can be effectively manipulated, but further research is needed to identify specific tools for tailoring them. It appears that protein composition of the samples may influence the functional properties, especially the 11S:7S protein ratio may play a determining role (Barac et al., 2010).

4. Conclusion

The results of this study indicate that pea seeds can be utilized to prepare protein-rich intermediates to be used in the production of foods. In case of 'Salamanca', PF, PPF, and PPI were found to be highly soluble at alkaline pH values. PPI has also a high solubility at pH 2. However, buffering capacity of these fractions is also quite intense. Air classification (PPF) and isoelectric precipitation (PPI) allowed the concentration of field pea protein by nearly 3- or 4-fold in comparison to PF. Furthermore, water binding capacity could be increased, while the trypsin inhibitor activity is reduced. This is quite interesting for food technology approaches. Also including the hulls of the peas is an option, but here the trypsin inhibitor activity increases dramatically. As already mentioned, it was confirmed that pea protein has a high buffering capacity over a pH range from 4–10. This is important for industrial processing because it influences the solubility and the functional behaviour of the proteins. These results further indicate that it is advisable to prepare a protein enriched fraction such as PPF rather than preparing protein isolates. Both, protein content and quality with regard to content of trypsin inhibitors can be improved. Further experiments are needed to

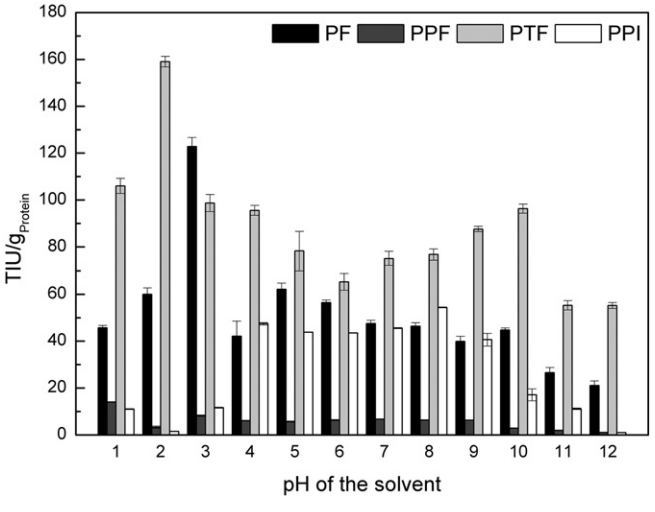

Fig. 6. Trypsin inhibitor content of the pea flour fractions. (PF: pea flour, PPF: protein-rich pea flour. PTF: pea testa flour, and PPI: pea protein isolate); n = 3.

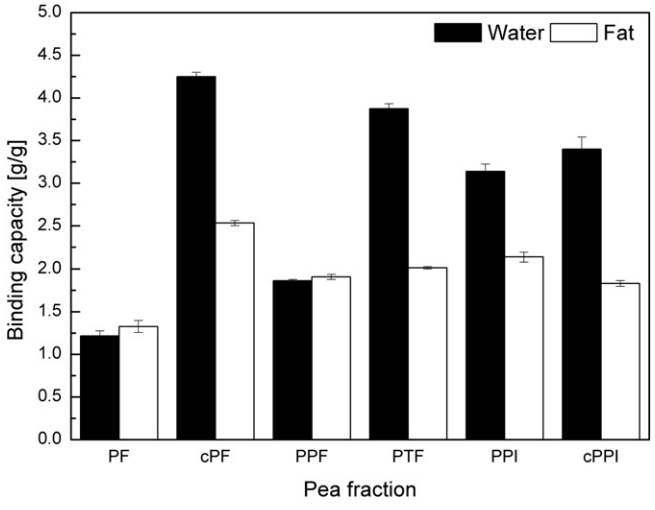

Fig. 7. Water and fat binding capacity of the pea fractions and products [g/g]. (PF: pea flour, cPF: commercial pea flour, PPF: protein-rich pea flour, PTF: pea testa flour, PPI: pea protein isolate, and cPPI: commercial pea protein isolate).

address bioavailability issues especially with regard to the distribution of amino acid profiles and bioavailability of indispensible amino acids. Finally, the potential of by-products (here: basis for PTF) need to be investigated further, as interesting proteins such as amylase inhibitors have also been identified in peas providing further options for functionalized added value products. Combining several physical separation methods such as air classification under different thermal conditions may provide a more suitable technique for preparing protein rich intermediates rather than extensive isolation procedures.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.05.009.

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4.1.3 Impact of thermal treatment versus cold atmospheric plasma processing on the techno-functional protein properties from *Pisum sativum* 'Salamanca'

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Impact of thermal treatment versus cold atmospheric plasma processing on the techno-functional protein properties from *Pisum sativum* 'Salamanca'

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ABSTRACT

This study aimed at evaluating the potential of cold atmospheric pressure plasma (CAPP) treatment for the functionalization of dry bulky and powdery food materials. CAPP treatment was capable of modifying protein and techno-functional properties of different flour fractions from grain pea (*Pisum sativum* 'Salamanca'). Experiments using a pea protein isolate indicated that the reason for the increase in water and fat binding capacities in protein rich pea flour to 113% and 116%, respectively, is based on plasma-induced modifications of the proteins as their solubility was increased to 191%. This is also supported by detected changes in tryptophan fluorescence spectra. With increasing treatment times the fluorescence emission intensity increased at 328 nm and decreased at 355 nm indicating structural and/or compositional changes of the proteins. The results indicate that the application of CAPP can be exploited as a means to modulate functionality of dry bulk materials in the food sector.

1. Introduction

Food engineering may also be described as the attempt to preserve, transform, create or destroy structures that have been imparted by nature or processing (Aguilera and Stanley, 1999). To fulfill the consumer demands, during the last century the food industry developed a multitude of products now available in the supermarket. Thereby, major advances in food engineering came from transfer and adaptation of knowledge from related fields such as chemical and mechanical engineering (Aguilera, 2005). In the past, the focus was largely at the processing or macroscopic level through the adaptation of unit operations and design of process equipment to transform and preserve foods. Further improvements on the quality of existing foods and the creation of new products to satisfy expanding consumer demands during this century will be based largely on interventions at the microscopic level, as the majority of elements that critically participate in transport properties, physical and rheological behavior, textural and sensorial traits of foods are below the 100 μm range (Aguilera, 2000). Among others, those microstructural elements substantially contributing to techno-functionality, food identity and quality are mainly fibers, small particulate material in powders, starch granules and protein assemblies (McClements, 2007). Particularly the tools and basic knowledge of food material science favors the change in scale of intervention and further shift the focus of the food industry from processes to products (Aguilera, 2005; Cussler and Wei, 2003).

Many food processing operations aim at modifying raw materials or intermediates in order to provide products with desirable traits and functional properties. For producing high quality consumables, intermediates and end products in powder or bulk form, efficient and high-performance processes are at least as important as the use of high-grade raw materials (Cuq et al., 2011). Surface modification using cold atmospheric pressure plasma (CAPP) is an effective and economical technique for many materials and of growing interests in food engineering, as it is quite difficult to design granular and powder products fulfilling both needs, adequate bulk properties followed by a special treatment to modify the surface properties (Chu et al., 2002; Förch et al., 2004; Höcker, 2002; Schröder et al., 2001). The surface-effects, such as plasma sputtering and etching, induced by applying CAPP to foodstuffs, may offer an innovative approach to enhance the surface and techno-functional properties selectively while the bulk attributes of the materials remain unchanged (Fricke et al., 2012, 2011; Schröder et al., 2001). Plasma gas is composed of highly excited atomic, molecular, ionic, and radical species and consists of a large number of reactive species such as electrons, positive and negative ions, free radicals, gas atoms, molecules in the ground

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or excited state, quanta of electromagnetic and UV radiation (photons) as well as visible light (Laroussi & Leipold, 2004). Plasma, the fourth state of matter, could be generated in a large range of temperature and pressure by means of coupling mechanical, thermal, nuclear radiant energy or carriers of an electric current to a gaseous medium (Conrads & Schmidt, 2000). Density and temperature of the electrons are altered depending on type of energy supply and amount of energy transferred to the working gas. High temperature plasma thereby implies that all species are in a thermal equilibrium state. Low temperature plasma is subdivided into thermal plasma (quasi-equilibrium plasma) being in local thermal equilibrium state and non-thermal plasma (non-equilibrium plasma) also called cold plasma (Schlüter et al., 2013). Low pressure glow discharge plasmas are of great interest in fundamental research but must be contained in costly air tight enclosures making them expensive and time consuming. Therefore, innovative plasma sources operating at atmospheric pressure by retaining the properties of low pressure media were developed (Kogelschatz, 2002,1999). Economic and operational advantages have led to the development of a variety of atmospheric plasma sources for several scientific and industrial applications. Thus, CAPPs have received a great deal of attention in the last two decades. CAPP may be obtained by a diversity of electrical discharges such as corona discharge, micro hollow cathode discharge, atmospheric pressure plasma jet, gliding arc discharge, dielectric barrier discharge or by radiofrequency (rf) and microwave. Due to its remarkable potential for being environment friendly and energy saving, its flexibility and capability for creating new products and its clear ecological advantages, enormous potential is attributed to the CAPP technology in a large number of diverse and unrelated fields in scientific and industrial areas.

It was shown, that the high-density of ionized and excited species in the plasma can change the surface properties of normally inert materials such as ceramics or glass (Jiang, 2005; Meyer-Plath et al., 2003; Taubert et al., 2013). In particular, modification of the surface energetics of the materials can improve the techno-functional bulk properties, as the flowability, compactibility, clumping, particle sphericity as well as the adhesion strength, surface and coating properties and could therefore contribute to improved handling, application and storage characteristics (Fitzpatrick and Ahrné, 2005; Spillmann et al., 2007; Watano et al., 2000). In recent years, the main objective of the plasma based research work is to ensure high microbial product safety and enzymatic stability by the application of CAPP under retaining the initial product quality (Fernández et al., 2013; Fröhling et al., 2012a; Hertwig et al.; Pankaj et al., 2013; Surowsky et al., 2013). Thus, CAPP also qualifies as a new discipline in food processing and has been considered as an emerging nonthermal technology for the improvement of food safety since it is capable of effectively inactivating a wide range of microorganisms including spores and viruses (Baier et al., 2014; Birmingham, 2004; Surowsky et al., 2014; Terrier et al., 2009). For this purpose, CAPP has been applied for the decontamination of raw agricultural products, egg surface and real food systems and is proved to have specific potential for treatment of foods (Schlüter et al., 2013). Furthermore, it was observed that, similar to the plasma application in material science, CAPP is capable of modifying wet and dry surfaces of agricultural and food stuff (Grzegorzewski et al., 2010; Khanal et al., 2014; Misra et al., 2015). Up to now, this unique feature is only used in the non-food sector. The technology transfer from those research fields and industrial branches to food science and technology may offer an innovative approach for the targeted modification and functionalization of powdery and bulky food surfaces.

Within the context of the protein crop strategy, emphasis is currently being placed on the sustainability, low cost and nutritional properties of plant-based proteins as an alternative to the established animal-based proteins that are currently in the market. Legumes, as peas, beans and lentils, historically been utilized mainly as whole seeds, constitute a promising alternative to the critically considered use of soy. However, in recent years, interest has grown in the utilization of legumes in other forms (e.g. like flour, concentrate, isolate) rather than the whole seeds (Doxastakis, 2000; Saio, 1993). Depending on the production process of alternative legume flour fractions their techno-functional properties are limited (Sun and Arntfield, 2010). For this purpose, a considerable amount of work has been accomplished in order to modify legume-based raw materials, intermediates and products while preserving their nutritional value.

Main objective of this study was to investigate the possible use of the CAPP technology for the modification of techno-functional properties and protein solubility of protein-rich, starch-rich, and fiber-rich fractions as well as of a protein isolate from grain pea (*Pisum sativum* 'Salamanca') and to contrast the obtained effects with those induced by a comparable thermal treatment.

2. Material and methods

2.1. Pea flour fractions

Grain peas (*P. sativum* 'Salamanca', Norddeutsche Pflanzenzucht, Hans Georg Lembke GmbH, Hohenlieth, Germany) with a crude protein content of 20% (Kjeldahl (§64 LFBG), $N = 6.25$) served as test material. Dry seeds were hulled using a shelling machine (F. H. SCHULE Mühlenbau GmbH, Reinbeck, Germany), finely ground and classified (CONDUX CSM 80 classifier mill, Erich Netsch GmbH, Hanau, Germany) into a protein-rich (PPF, crude protein content 48.3%, Kjeldahl (§64 LFBG), $N = 6.25$) and a starch-rich (PSF, crude protein content 15.3%) pea flour fraction characterized by a maximum particle size of 500 µm (MEZ, Prag, Czech Republic). Pea testa flour (PTF, crude protein content 2.8%, Kjeldahl (§64 LFBG), $N = 6.25$) was prepared using a centrifugal mill (Ultra Centrifugal Mill ZM 200, Retsch, Haan, Germany). Pea protein isolate (PPI) was recovered from PPF by extraction with distilled water (1:8 w/v) adjusted to pH 1.5 (concentrated hydrochloric acid) under stirring (300 rpm) at room temperature for 30 min. Extract was centrifuged (Megafuge 2.0 R, Heraeus Sepatech GmbH Dusseldorf, Germany) at 4000 g and 4 °C for 60 min. The clear supernatant was collected and proteins were precipitated by adjusting the pH to 4.5 (1 M sodium hydroxide solution). Following freeze drying (Christ Alpha 1–4 Gefriertrocknungsanlage, Christ Gefriertrocknungsanlagen Osterode, Germany) and grinding (Ultra Centrifugal Mill ZM 200, Retsch, Haan, Germany) of the precipitate, the PPI (crude protein content 81.2%, Kjeldahl (§64 LFBG), $N = 6.25$) was stored at −20 °C until CAPP treatment.

2.2. Cold atmospheric pressure plasma (CAPP) treatment

For semi-direct CAPP treatments of dry bulk materials, a surface dielectric-barrier air-discharge (SDBD) system similar to that described by Oehmigen et al. (2010) was used. The SDBD plasma source consists of an array of 7 concentric ring-shaped electrodes (85 mm outer diameter) embedded in a 1.5 mm thick epoxy-glass bulk material mounted into the upper shell of a petri dish (90 mm diameter). Plasma treatments were performed in a cylindrical reaction chamber (15 cm height and 10 cm inner diameter), which surrounds the sample holder mounted on a height adjustable rotary shaker (JD 20, JVM Antriebe, Jöst, Dülmen, Gernamy). The SDBD plasma source was screwed in the cover of the reaction chamber assuring a constant installation position during the entire tests. Further details regarding the plasma source set-up can be found elsewhere (Bußler et al., 2015). In order to

prevent interactions between plasma-immanent species and ambient atmosphere, the airtight treatment chamber was filled with ambient air and hermetically sealed during plasma ignition. CAPP was generated at the surface of the dielectric epoxy glass by applying a sinusoidal voltage of 8.8 kV_{PP} at a frequency of 3.0 kHz delivered by a commercial function/arbitrary waveform generator (max. 20 MHz, DG1022, Rigol, Puchheim, Germany). The actual voltage was controlled via a built-in two channel digital storage oscilloscope (max. 500 MHz, TDS 2001C, Tektranx, Beaverton, USA).

For all trials, CAPP treatment of 4.75 g of each respective powder was conducted in an empty Petri dish, which was fixed on the holder of the shaker under the plasma source at a distance of 12 mm. To ensure homogeneity of treatments, thin layers of powder evenly spread over the base area (50.3 cm^2) of the Petri dish under the plasma source were agitated continuously (350 rpm) on the rotary shaker during exposure to CAPP for up to 10 min. CAPP treatments were conducted in triplicate.

2.3. Thermal treatment

Thermal treatment of the bulk materials was carried out in a drying cabinet ensuring a bulk temperature profile according to that recorded during exposure to CAPP. For this purpose glass Petri dishes (base area 50.3 cm^2) were preheated to 40 °C subsequent to addition of the bulk material followed by heating to a maximum of 60 °C over a treatment time of up to 10 min. Temperature was recorded as described above and temperature of the drying oven was adjusted manually. After 1, 2.5, 5, 7.5 and 10 min thermal treatment was broken off by removing the bulk materials from the drying cabinet and transferring them into a cooled Petri dish.

Due to the significant plasma-induced pH shift in protein extracts recovered from the PPI fraction, thermal treatment of the PPI was combined with adjusting the pH of the protein extracts according to that measured during extraction of proteins from CAPP treated PPI in an additional test series.

2.4. Temperature, mass loss, pH and dry matter content

During CAPP application to the dry bulk materials, bulk temperature increase was measured with a fiberglass-encased optic thermocouple (K-type) inserted through an optional opening of the treatment chamber. The thermocouple was positioned amid the bulk at the bottom of the Petri dish. At a frequency of 0.1 Hz, results were recorded with a USB data acquisition system (Personal Daq/56, SynoTECH, Hückelhofen, Germany) and the DASYLab 13.0 software for 10 min. The pH values of the protein extracts were measured by an Inolab Terminal 740 pH measurement device (WTW, Weilheim, Germany). Plasma-induced mass loss of the samples was determined by weighing the bulk before and after exposure to CAPP and thermal treatment, respectively. Dry mass (DM) of the bulk materials was obtained after oven-drying at 105 °C for 48 h and their water content was calculated from initial and dry mass.

2.5. Color measurement

The HunterLab-system was used to measure potential impact of CAPP and thermal treatment on the color of bulk materials immediately after the respective treatment and during storage over a period of 42 days. A Minolta spectrophotometer (CM-2600D, Konica Minolta Inc., Osaka, Japan) was set at illuminant D65, 3 mm aperture, and 0° viewing angle. *L*-value (brightness), *a*-value (green–red axis), and *b*-value (blue–yellow axis) were taken for nine samples of each different plasma exposure times. Change in color was calculated as ΔE following Eq. (1) whereas the indices 0 and *p* indicate measured values prior to and following thermal or CAPP treatment.

$$\Delta E = \sqrt{(L_0 - L_p)^2 + (a_0 - a_p)^2 + (b_0 - b_p)^2} \quad (1)$$

2.6. Water (WBC) and fat binding capacity (FBC)

The method by Smith (1978a, 1978b), modified by Quinn and Paton (1979) was used for determining the WBC of the pea flours. The required amount of water was calculated by the method of Smith (1978a), modified by Schwenke (1981). Therefore 0.5 g (±0.009 g) of the pea flour were weighted into a centrifuge beaker and stirred (60 s) with 2.5 mL of water using a propeller stirrer and an overhead agitator (Yellowline®, IKA® OST basic, New Jersey, USA). After 15 min of centrifugation at 3900 g the supernatant was decanted and the beaker was put upside-down on fiber paper for 60 min and was weighed. WBC was calculated as:

$$((F - I)/I_{DM}) * 100\% \quad (2)$$

Whereby, *I* is the initial weight of the sample, *F* is the final weight of the sample and I_{DM} is the initial weight of the sample based on dry mass determined as described in Section 2.3.

For the determination of the FBC, the method by Schwenke (1981) was used. 0.5 g (±0.009 g) of the respective pea flour fraction were weighed into a centrifuge beaker and stirred with 5 mL of commercial rape seed oil using a propeller stirrer and an overhead agitator (Yellowline®, IKA® OST basic, New Jersey, USA) two times for 60 s at 1000 rpm with a five-minute intermission in between (the stirring steps). After 20 min of centrifugation at 3900 g the supernatant was decanted and the beaker was put upside-down on fiber paper for 60 min and was weighed. Similar to WBC, FBC was calculated following Eq. (2).

2.7. Protein solubility

Following CAPP treatment 0.5 g (±0.0009) of the respective pea flour fraction were weighed into a small beaker. Extraction was carried out at ambient temperature using distilled water (PPF and PPI) and 0.1 M borate buffer pH 9 (PPI) under stirring on a rotary shaker (350 rpm). Protein extracts were centrifuged for 10 min (10,000 g, 4 °C) and subsequently analyzed (Day 1). Storage experiments were carried out in sealed Petri dishes under exclusion of light at ambient temperature over a period of 42 d. Seven, fourteen, twenty-eight and forty-two days after treatment extraction samples were taken from the bulk materials and analyzed.

The Biuret assay (Robinson and Hogden, 1940) was used for quantitative protein analysis. Biuret reagent was prepared by dissolving 1.5 g of copper sulfate monohydrate (Merck, Darmstadt, Germany) and 6.0 g of potassium sodium tartrate (Roth, Karslruhe, Germany) in 500 mL of distilled water. 300 mL of 10% (w/v) sodium hydroxide solution (Merck, Darmstadt, Germany) were added and the solution was filled up to 1000 mL with distilled water.

Bovine serum albumin (Fluka, Buchs, Switzerland) was used as a standard (c_{Biuret} = 0–10 mg/mL in 2 mg/mL intervals). The assay consisted of 200 µL of the protein extracts reacting with 800 µL of Biuret reagent (45 min, 20 °C). The absorption maximum at 540 nm was measured against a blank value (solvent) by UV/Vis spectrophotometer (BioPhotometer plus, Eppendorf, Hamburg, Germany). Yield of soluble pea protein was related to the dry matter and crude protein content of the respective pea flour fraction.

2.8. Fluorescence measurement

Measurements of fluorescence emission spectra were performed using a PerkinElmer LS55 fluorescence spectrometer (Rodgau-Jügesheim, Germany) equipped with a pulsed xenon lamp and a red-sensitive photomultiplier (R928). The excitation wavelength was 280 nm and the fluorescence spectra were scanned in a wavelength range of 300–550 nm. A cut-off filter at 290 nm was placed in front of the emission monochromator (slit width 5). Differences in protein concentration and pH of the samples were equalized by dilution with 0.1 M phosphate buffer (pH 7). Measurement was conducted in triplicate using disposable semi-microcuvettes (Sarstedt Nümbrecht, Germany) against phosphate buffer as the blank.

3. Results and discussion

3.1. Impact of CAPP treatment on thermal load, dry matter and mass loss of the bulk materials

During exposure of the bulky pea flour fractions to CAPP a continuous temperature increase from ambient temperature (22 °C) to up to a maximum of 59.8 °C occurred (Fig. 1), which can be attributed to the heat up of the plasma source during plasma ignition and the resultant insufficient convectional heat dissipation due to the short distance of 12 mm between sample and surface of the plasma source. In most studies concerning the plasma application on food surfaces or model food systems, plasma jet systems were used leading to a gradual reduction in sample surface temperature, due to the flow of the plasma working gas and evaporation effects from moist product surfaces (Fröhling et al., 2012b; Surowsky et al., 2013). Consequently, the low moisture content of the samples in this study prevents the cooling of the sample surface by the evaporation of water and in turn leads to a higher thermal load of the bulk material. This presumption is confirmed by the fact, that dry matter of the bulk materials was neither influenced by exposure to CAPP nor by thermal treatment. The initial dry matter contents of 91.73 (±0.39)% for PPF, 91.39 (±0.30)% for PSF, 92.44 (±0.47)% PTF and 91.27 (± 0.47)% for PPI, respectively, were not significantly influenced (data not shown). Nonetheless, significant plasma-induced mass losses were detected (Table 1) amounting to a maximum of 2.1% for PPF, 1.2% for PSF, 1.3% for PTF and 1.1% for PPI, respectively. Moreover, a clear correlation between the plasma exposure time and the mass loss showed up, which in turn was found to be specific to the pea flour fraction treated and did not occur after applying an appropriate thermal treatment to the bulk materials. Hence, obtained mass loss can be traced back to plasma-specific interactions with the particle surfaces of the bulk materials treated. Similar effects were demonstrated by Khanal et al. (2014), who evidenced the ablation of cuticular membrane material from apple resulting in significant mass losses, and Grzegorzewski et al. (2010), who proved the removal of cuticular wax from the surface of lamb's lettuce leaves. Plasma-induced erosion, sputtering and ablation phenomena by high-energy particles were determined in numerous investigations for a wide range of plasma sources and materials (Petrik and Kimmel, 2004) whereas the main mechanisms of ablating surfaces are considered to stem from ion bombardment and production of chemically active species that oxidize organic compounds. Due to the high etch rate, argon and oxygen plasmas were found to be particularly useful in ablating polymers (Fricke et al., 2011). As optical emission spectra of the CAPP-source used in this study was dominated by UV-A and UV-B radiation (Bußler et al., 2015) and not by vacuum UV (VUV) radiation, as a characteristic compound in low pressure plasmas, known VUV-induced oxidation and ablation phenomena (Fozza et al., 1997) can be excluded as possible causes for mass loss in this study.

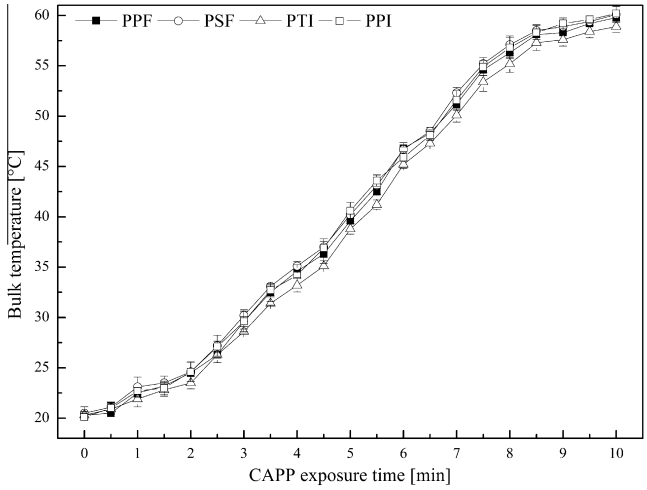

Fig. 1. Thermal bulk load of PPF, PSF, PTF and PPI during exposure to CAPP (3.0 kHz, 8.8 kVpp) for 10 min.

3.2. Effect on product color

Neither thermal nor CAPP treatment led to a change in product color recognizable by the human eye. Color measurement using the HunterLab-system also revealed no changes in color following thermal treatment and during storage (data not shown). CAPP treatment in turn slightly influenced the product color indicated by a slight increase in ΔE from 2.4 to 4.5 and 3.5 to 5.0 by exposing PPF and PPI to CAPP for 1 to 10 min (Table 2). Regarding the PSF most intensive change in color (9.3) occurred after exposure to CAPP for 1 min further declining to 8.9 with extending the exposure time to 10 min. All measured color differences remained stable over storage for 42 d (data not shown).

3.3. Modification of water (WBC) and fat binding capacity (FBC)

As depicted in Fig. 2 water and fat binding capacities of the flour fractions differed significantly. While the WBC of PPF and PSF were found to be in the range of 1.95 g/g and 2.0 g/g, WBC of the PTF was

Table 1
Means (n = 3, ±sd) of mass loss of the pea flour fractions induced by CAPP and thermal treatments related to the initial weights of the samples.

Mass loss (%)								
Treatment time (min)	Thermal treatment				CAPP treatment			
	PPF	PSF	PTF	PPI	PPF	PSF	PTF	PPI
0	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)
1	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.4 (±0.1)	0.1 (±0.0)	0.5 (±0.1)	0.3 (±0.0)
2.5	0.1 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	0.0 (±0.0)	1.1 (±0.2)	0.4 (±0.1)	1.3 (±0.2)	1.1 (±0.1)
5	0.2 (±0.0)	0.1 (±0.0)	0.0 (±0.0)	0.1 (±0.0)	1.3 (±0.2)	0.6 (±0.1)	1.5 (±0.3)	1.4 (±0.1)
10	0.2 (±0.0)	0.1 (±0.0)	0.1 (±0.0)	0.1 (±0.0)	2.1 (±0.3)	1.2 (±0.2)	2.2 (±0.3)	2.0 (±0.2)

Table 2
Means ($n = 9$, ±sd) of thermal and CAPP-induced change in color of PPF, PSF, PTF and PPI calculated as ΔE.

ΔE (–)								
Treatment time (min)	Thermal treatment				CAPP treatment			
	PPF	PSF	PTF	PPI	PPF	PSF	PTF	PPI
1	0.1 (±0.0)	0.1 (±0.1)	0.0 (±0.0)	0.0 (±0.0)	2.4 (±0.1)	0.9 (±0.1)	9.3 (±0.4)	3.5 (±0.1)
2.5	0.1 (±0.0)	0.2 (±0.0)	0.1 (±0.0)	0.0 (±0.0)	3.7 (±0.2)	0.6 (±0.0)	9.3 (±0.3)	3.9 (±0.1)
5	0.2 (±0.1)	0.2 (±0.1)	0.1 (±0.0)	0.1 (±0.0)	4.3 (±0.2)	0.5 (±0.1)	9.1 (±0.3)	4.3 (±0.1)
10	0.2 (±0.0)	0.3 (±0.0)	0.0 (±0.0)	0.1 (±0.0)	4.5 (±0.1)	0.3 (±0.0)	8.9 (±0.4)	5.0 (±0.2)

Table 3
Means ($n = 9$, ±sd) of actual pH values in protein extracts recovered from thermal and CAPP treated PPI (1:25 w/v in distilled water and 0.1 M borate buffer, pH 9).

pH of the protein extract (–)							
Solvent	Initial pH (–)	CAPP exposure time (min)					
		0	1	2.5	5	7.5	10
Dist. water	6.6	4.0 (±0.02)	4.0 (±0.02)	3.8 (±0.02)	3.6 (±0.02)	3.5 (±0.02)	3.4 (±0.02)
Buffer	9	5.6 (±0.02)	5.5 (±0.02)	5.2 (±0.02)	4.9 (±0.02)	4.7 (±0.02)	4.6 (±0.02)

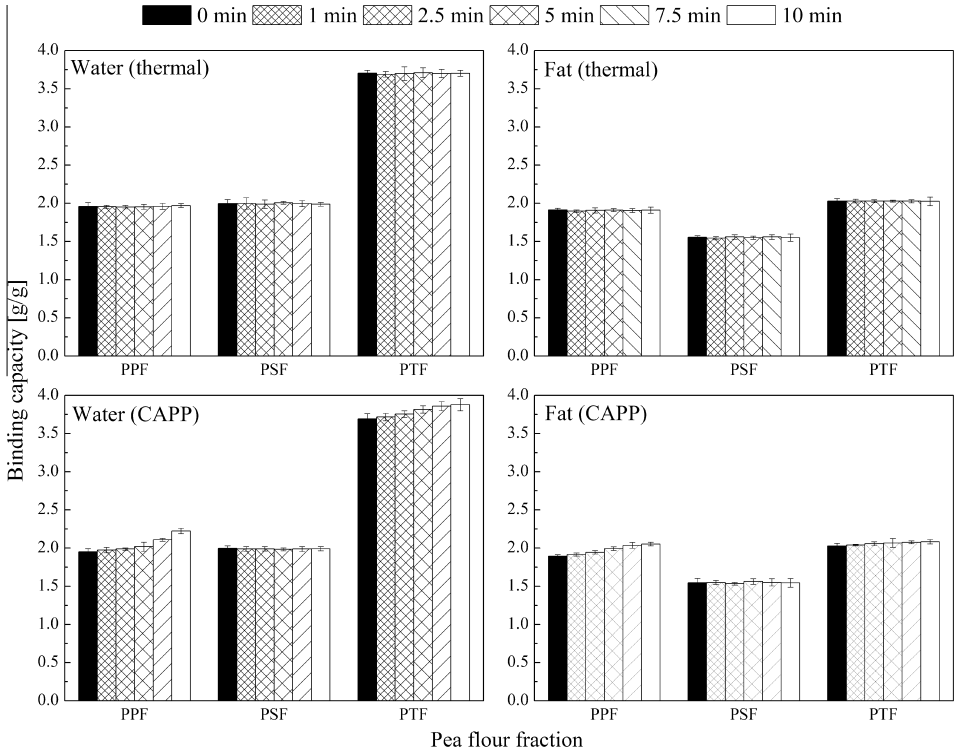

Fig. 2. Impact of thermal (top, 60 °C, up to 10 min) and CAPP (bottom, 3.0 kHz, 8.8 kVpp, up to 10 min) treatment on water and fat binding capacities of PPF, PSF and PTF.

significantly higher (3.7 g/g). In contrast FCB of the PPF (1.9 g/g) was similar to that of the PTF (2.1 g/g). FBC of the PSF (1.6 g/g) was significantly lower. Both, water and fat binding capacities were not influenced by thermal treatment of the bulky pea flour fractions. Further the CAPP treatment of the PSF did neither lead to a change in WBC nor in FBC of the PSF. Regarding the CAPP treatment of PPF and PTF, water and fat binding capacity was slightly modified which was demonstrated in a continuous increase in WBC to 113% (PPF) and 106% (PTF), respectively, by exposing the flour fractions to CAPP for up to 10 min. For those two flour fractions exposure to CAPP also featured a slight increase in FBC to 116% (PPF) and 105% (PTF), respectively. Consequently, the obtained results demonstrate that the plasma-induced effects on water and fat binding capacities were dependent upon exposure time and composition of the matrix treated. Plasma-product interactions and resulting surface-modifying characteristics were more pronounced for high-protein and high-fiber matrices whereby they were not apparent or not detectable regarding the starch-rich fraction. These findings appear to be consistent with those concerning the plasma-induced mass loss of the bulky flour fraction in this study. In contrast, Zou et al. (2004) reported the modification of starch in an argon glow discharge plasma that was manifested in a loss of OH groups which is probably due to the cross-linking of α-D-glucose units. Potato and corn starch experienced a degree of destruction to the supramolecular and molecular characteristics which the authors attributed to the fracture of glycoside bonds in the solid starch granules close to the center of molecular chain and also discussed the destruction of the starch structure due to

the induction of higher amount of inter-helical water molecules in each of the crystal units of the B-type crystalline structure, which could be induced by oxygen generated plasma to become free radicals or other active species. However, with the analytical methods applied in this study, no indications could be observed regarding the modification of starch granules.

Regarding interactions between plasma and proteins, it is a well-known fact, that proteins and proteinaceous matters are degraded due to the impact of atomic oxygen playing the dominant role in degradation reactions (Deng et al., 2007). A potential synergistic effect of nitric oxide contributing to the decomposition and minor roles for UV photons, OH radicals and O_2 metastable states have been identified (Perni et al., 2007). Using a wide range of physical techniques including SEM images, EDX analysis, electrophoresis experiments, fluorescence spectroscopy, and inactivation kinetics, Deng et al. (2007) demonstrated that plasma-treated proteins were either removed from a stainless steel surface as fragments or damaged significantly if remained on the surface. Plasma-induced chemical protein modification on the surface of the PPF in this study may have contributed to an alteration in hydrophilic/hydrophobic surface characteristics and in turn to the modified water and fat binding capacities.

Obtained effects regarding the PTF may be caused by chemical reactions of highly reactive plasma-immanent species that result in creation of excessive double bonds, production of low mass stable degradation products, large crosslinked structures and eventually oxidized structures (Berlett and Stadtman, 1997). It is known from previous comparable studies in the field of material science, that CAPP treatment of non-polar polyolefins results in creation of polar groups on the polymer surface enhancing printability, wettability, adhesion with inorganic materials or with biologically active components (Hegemann et al., 2003). Further, a twofold effect of plasma treatment on the surface of wool was reported: oxidation and partial removal of the hydrophobic lipid layer on the wool surface and oxidation the disulfide bonds and reduction of the cross-link density, as the exocuticle, that is the layer of the surface itself (epicuticle), is highly cross-linked via disulfide bridges. As the surface is oxidized, the hydrophobic character is changed to become increasingly hydrophilic (Sparavigna, 2008). Similarly, the outermost layer of the legume seed coat is the waxy cuticle (Moïse et al., 2005); oxidation and partial removal of the hydrophobic lipid layer, oxidation of the disulfide bonds and reduction of the cross-link density on the surface are also conceivable here. The removal of epicuticular waxes from plant surfaces by CAPP and the resulting increase in surface wettability has been reported by Grzegorzewski et al. (2010) and Khanal et al. (2014) and hence has to be taken into consideration as a potential cause of the effects obtained in this study.

3.4. Impact on pH, protein solubility and fluorescence emission spectra

Thermal treatment did neither significantly influence the pH of the protein extracts recovered from the PPF subsequent to treatment nor during the storage period of 42 d (Fig. 3). The decrease in pH from 9 to about 8.7 can be attributed to the high buffer capacity of the contained soluble pea proteins. In contrast, exposure to CAPP did significantly reduce the pH of the protein extracts from 8.4 (0 min) to 8.3 (1 min), 8.1 (2.5 min), 8.0 (5 min), 7.9 (7.5 min) and 7.6 (10 min) subsequent to the treatment. pH decrease tended to be dependent on treatment time as it was more pronounced after increasing the exposure time to CAPP. Upon storage pH further decreased to 8.3 (0 min), 8.2 (1 min), 8.1 (2.5 min), 7.9 (5 min), 7.7 (7.5 min) and 7.2 (10 min), respectively. Regarding the protein extracts recovered from PPI using distilled water as solvent (pH 6.6) the pH dropped to 4.0 during extraction of the untreated PPI (Table 3). This is most likely attributed to the high buffer capacity of the soluble pea proteins in the range of pH from 3 to 10 (Reinkensmeier et al., 2015). Exposing the PPI to CAPP lead to a pH drop to 4.0 (1 min), 3.8 (2.5 min), 3.6 (5 min), 3.5 (7.5 min) and 3.4 (10 min), respectively, during solvation of the PPI. This corresponds to a pH drop of Δ0.7 (10 min). In buffer (pH 9) plasma-induced pH drop was in the range of 1.0 (10 min, from pH 5.6 to 4.6) and, thus, even more pronounced as in distilled water. For both solvents pH reduction increased with extended exposure of the bulk material to SDBD plasma prior to protein solvation. In literature, the plasma-induced decrease in pH has often been reported for liquid matrices. After an indirect surface DBD treatment of different liquid volumes Oehmigen et al. (2010) reported a steep decrease of pH from 7 to less than 4 with non-buffered physiological saline within the first 5 min of plasma treatment, followed by a slight further decrease reaching more or less stable pH values between 2 and 3 within 30 min dependent on sample volume. Similar results were reported by Helmke et al. (2009). In atmospheric pressure air plasmas generation of reactive oxygen species (ROS) can be anticipated just as the generation of reactive nitrogen species (RNS) like NO and NO*x* (Laroussi & Leipold, 2004). Consequently, acidification of non-buffered solutions could be interpreted as a consequence of the formation of nitrous acid (HNO_2) and nitric acid (HNO_3) from NO via NO_2 (Doubla et al., 2008; Sakiyama et al., 2009). Currently, here is no data available concerning the plasma impact on pH of dry solid systems. Acidification of solvents in this may be attributed to the transition of non-covalently bound reactive plasma species or even split-off protein parts or amino acids into the medium that either cause the acidic character or further may lead to the formation of acidic compounds.

Solubility of the proteins contained in the PPF showed up in the range of 74% at pH 9 (Fig. 3). Thermal treatment did not lead to any significant changes in protein solubility subsequent to treatment except for a treatment time of 10 min which led to a slight decrease in protein yield to about 68% further decreasing to 63% over a storage period of 42 d. In contrast initial protein solubility of 71% (0 min) was strongly influenced by CAPP treatment resulting in decreased protein yields of 70% (1 min), 62.5% (2.5 min), 39% (5 min), 35% (7.5 min) and 33% (10 min), respectively. During storage of the PPF protein solubility further decreased except for an exposure to CAPP for 1 min.

Protein solubility of the PPI (Fig. 4) was found to be very low and ranged from 11.5% in distilled water to 6% in buffer (pH 9). Exposure to CAPP also significantly influenced the solubility of the pea proteins. In distilled water content of soluble proteins was increased with increasing exposure to CAPP. Solubility rose to 109% (1 min), 120% (2.5 min), 126% (5 min), 157% (7.5 min) and 191% (10 min), respectively, when relating the content of soluble pea protein to the untreated sample. Adjusting the pH of the PPI extract to 3.6 (corresponding to the pH of the protein extract of PPI exposed to CAPP for 10 min), did not yield in a comparable protein content in the recovered extract. Instead, protein solubility was increased to 217% compared to the untreated sample (pH 4.0). Taking into account the thermal load of the PPI bulk during exposure to CAPP by thermal treatment of PPI at 60 °C for 10 min prior to protein extraction and pH adjustment of the suspension to 3.6 during protein solvation did not result in a change in protein solubility. In contrast, protein solubility using buffer as solvent was significantly lower (5.5%) compared to solvation in distilled water and further slightly decreased upon extended exposure to CAPP (4%, 10 min). Adjusting the pH of the PPI extract to 5.6 (corresponding to the pH of the protein extract of PPI exposed to CAPP for 10 min), in contrast lead to an increase in protein solubility to 381% compared. The combination of the thermal and plasma-induced pH conditions according to the CAPP treatment lead to an increase in protein solubility to 327%.

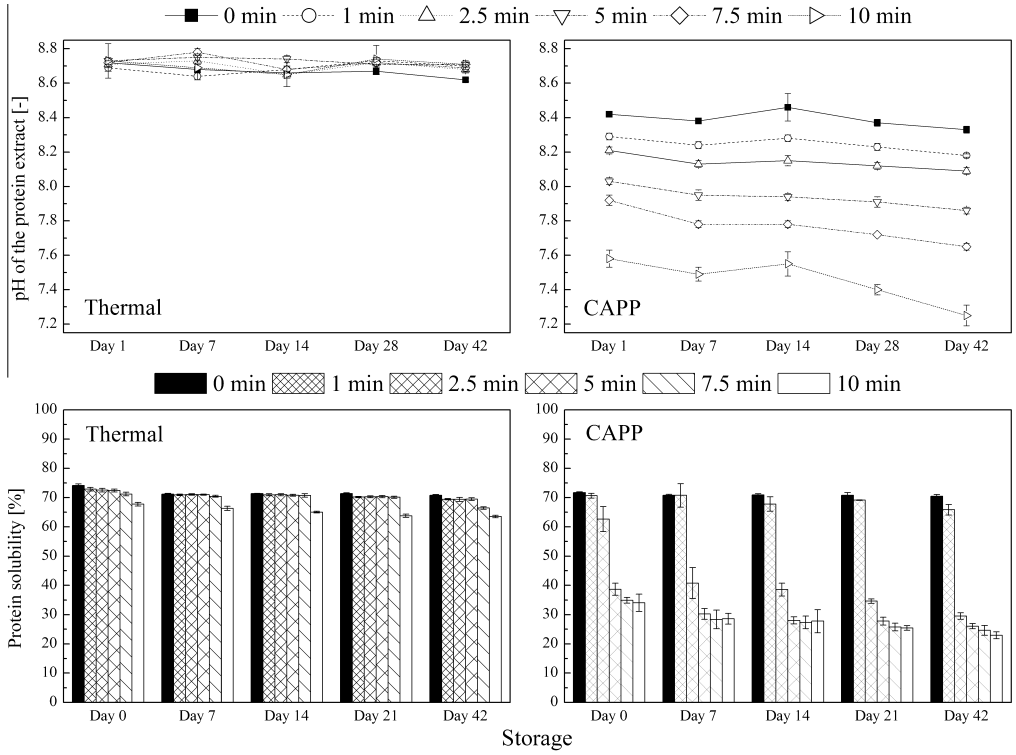

Fig. 3. Actual pH values in protein extracts recovered from thermal and CAPP treated PPF (1:25 w/v in 0.1 M borate buffer, pH 9) over a storage time of 42 d (top) and impact of thermal (bottom left, 60 °C, up to 10 min) and CAPP (bottom right, 3.0 kHz, 8.8 kVpp, up to 10 min) treatment on solubility of proteins recovered from PPF (0.1 M borate buffer, pH 9) at ambient temperature over a storage time of 42 d.

Up to now, protein-plasma interactions have hardly been scientifically researched. Possible underlying mechanisms are multifarious and need to be investigated in more detail. Plasma-immanent species as OH radicals can cleave peptide bonds and oxidize amino acid side chains. O radicals are involved in etching processes and the oxidation of proteins (Surowsky et al., 2014). Further the cleavage of disulfide bonds within a peptide due to dissociative addition of a hydroxyl radical to form RSH and RSO• at the cleavage site are conceivable. The work of Xia and Cooks (2010) supports the fact that disulfide bonds are homolytically cleaved with oxygen and hydrogen added onto each sulfur atom. Studies conducted by Surowsky et al. (2013), who reported the inactivation of polyphenol oxidase and peroxidase in aqueous model food systems consisting of a polysaccharide gel, revealed the occurrence of changes in the enzymes' secondary structure. This was also demonstrated for non-aqueous systems by Misra et al. (2015) revealing the alteration of the secondary structure of gluten proteins in wheat flour via FTIR spectroscopy following CAPP treatment. Furthermore, reactive oxygen species (ROS), as atomic oxygen or OH radicals, may attack for instance aromatic amino acids like tryptophan, which are sensitive to oxidation. The aromatic amino acids tyrosine, tryptophan and phenylalanine can also be found in pea proteins (Kuo et al., 2004; Pownall et al., 2010). As particularly tryptophan emits light in the region between 300 and 350 nm at an excitation wave length of 280 nm, changes in tryptophan fluorescence can be used as an indicator of oxidation reactions and (subsequent) changes of the conformation and three-dimensional structure of proteins (Gießauf et al., 1995; Vivian and Callis, 2001). In this study, fluorescence spectra of PPI solutions excited at 280 nm were characterized by an emission maximum at 328 nm and a second peak at 355 nm (Table 4). Different protein fluorescence maxima (and quantum yields) are most likely caused by various ratios of two or more discrete classes of tryptophan residue contained in pea protein which has to be considered as a mixture of several protein components (vicilin, legumin, albumin). Tryptophan residue classes detected in this study may include tryptophyls inside the protein in a low-polar hydrophobic microenvironment as well as tryptophyls on the surface of a protein in a high-polar aqueous microenvironment. Following Konev (1967), the former class is characterized by a short-wavelength position of the fluorescent maximum (λ = 331 nm), while the latter is characterized by a large Stokes shift (λ = 350 nm). Burstein et al. (1973) also reported that tryptophan residues in the core of proteins emit light in lower wavelength regions than those located at the surface. Fluorescence spectra determined in this study indicate the occurrence of structural changes of the pea proteins upon exposure to CAPP, as the fluorescence emission intensity increased at 328 nm and decreased at 355 nm with increasing treatment times (Table 4). Due to its aromatic character, tryptophan is often located in the hydrophobic core of protein interiors, at the interface between two protein domains/subdomains, or at the subunit interface in oligomeric protein systems. These side chains become more exposed to solvent upon disruption of the protein's tertiary or quaternary structure. The resulting change of tryptophan surroundings to a more polar environment (Carvalho et al., 2003) can be one of the reasons for the observed losses of fluorescence emission intensity at 355 nm, since quenching can affect the tryptophan and tyrosine fluorescence (Lakowicz, 2006). Plasma inherent reactive nitrogen species are also able to act as quenchers. Further, in a hydrophobic environment, such as in the interior of a folded protein, tryptophan emission occurs at shorter wavelengths which must be taken into consideration regarding the increase in fluorescence emission intensity at 328 nm with increasing exposure to CAPP. Besides the occurrence of quenching effects, the oxidation of tryptophan during plasma treatment as well as combinations of oxidation

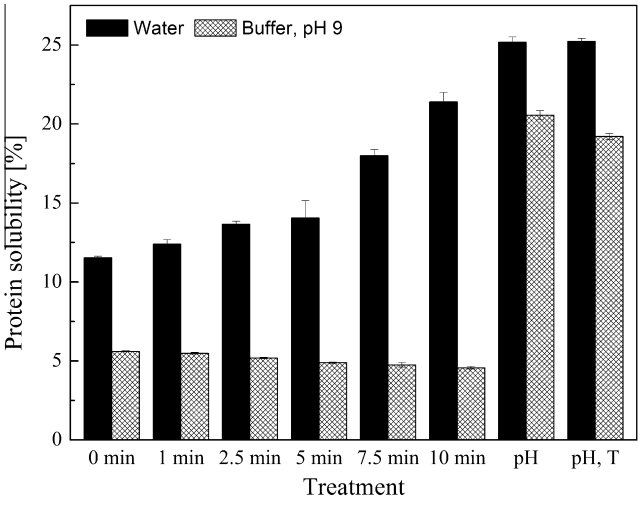

Fig. 4. Impact of CAPP (3.0 kHz, 8.8 kVpp, up to 10 min) treatment on solubility of PPI in distilled water and 0.1 M borate buffer (pH 9) at ambient temperature.

Table 4
Means ($n = 9$, ±sd) of thermal and CAPP-induced change in fluorescence emission intensity of aromatic amino acids in PPI ($\lambda_{ex} = 280$ nm, slit width 5, $\lambda_{em(max)} = 328$ nm, $\lambda_{em} = 355$ nm) dissolved in water. All differences in means are statistically significant ($p < 0.05$).

Treatment Plasma exposure time (min)	Fluorescence emission intensity at 328 nm (rel. units)	St. dev.	Fluorescence emission intensity at 355 nm (rel. units)	St. dev.
0	85	±0.4	52	±0.4
1	90	±0.3	51	±0.5
2.5	90	±0.5	49	±0.3
5	92	±0.2	48	±0.4
7.5	96	±0.4	45	±0.5
10	99	±1.1	44	±0.1
pH (–)/temperature (°C)				
3.4/20	43	±0.8	95	±0.4
3.4/60	57	±0.4	76	±0.2

reactions and quenching phenomena can be possible reasons. The results of the fluorescence measurements support the occurrence of plasma-specific structural changes of the pea proteins providing the most likely explanation for the observed plasma-induced effects on the solubility of pea proteins and on the techno-functional properties of the pea flour fractions. This assumption is further supported by the results obtained regarding the change of protein fluorescence in PPI exposed to the thermal and/or plasma-induced pH conditions which indicated structural and/or compositional changes of the proteins different to those obtained upon CAPP treatment.

4. Conclusion

This study shows the potential of CAPP application on dry bulk materials by maintaining their outer appearance. It was evidently ascertained that plasma-induced modifications of biomaterials' surfaces is transferrable to dry bulk food matrices. Particularly concerning the modification of protein and fiber rich matrices CAPP could provide an alternative nonthermal approach in the processing of bulky and powdery materials. Fluorescence measurements provided first indication of structural changes of the contained proteins, which are most likely a causal factor for the plasma-induced effects on protein and techno-functional properties. However, in particular underlying mechanisms need to be further investigated by using model systems and food matrices to identify the plasma-induced chemical and structural modifications especially focusing on proteins and fibers as the complexity of plasma chemistry though makes the explicit elucidation of the underlying reaction pathways a challenging and up to date not fully resolved task. The application of CAPP as one of the eco-friendly technologies, which can be applied to regulate the structure and properties of natural polymers, is expected to be promoted for protein and fiber polymer modification by the results of this work. In order to make CAPP a unique tool for the targeted modification of dry bulk materials a bunch of product-process interactions need to be taken into consideration for tailor-made process designs.

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4.2 Cross-value chain transfer of knowledge: processing of edible insects

Edible insects have gained attention in Europe as an underexploited sustainable protein and nutrient source for food and feed. On the one hand, the FOA pointed out their potential for example in two FAO publications [159, 160], on the other hand, a risk profile published by the EFSA [161] has emphasized the numerous uncertainties and knowledge gaps regarding the use of insects and products thereof as food and feed. In addition, consumer acceptance of insects in food products is a major challenge [162]. As it is higher with decreasing visibility of the insects [163], it is favorable to introduce insects to the human consumer in a masked form, as powder, meal or fraction. Extracting insect proteins for human food products – a process already being carried out [164] – could be a useful way of increasing acceptability among wary consumers. However, supplementing food products with insect-based hemi-products/ingredients, proteins and fractions requires extensive knowledge on their properties. Also in case of insect proteins, these properties include, among others, solubility, amino acid profile, thermal stability and techno-functional properties, as water and fat binding, gelling, foaming and emulsifying capacity. The targeted application of insect-derived ingredients in food formulae is facilitated by insect processing and protein extraction, as well as by the development of processes for the tailored modification of the respective functional properties [165, 166]. For an industrial bio-fractionation, established processing chains for the production of high quality and affordable proteins from traditional protein sources need to be adapted to meet the specific requirements of edible insects as a raw material. In this context, the first step for process adaptation or development must be the characterization of the fractions and proteins from respective potential insect species used for food and feed. The impact of processing steps under varying extraction conditions applied during recovery of protein concentrates and isolates on the protein properties need to be analyzed in order to investigate how the composition and properties of recovered insect flour fractions are affected. This includes identifying the necessary process stages and parameters required in order to maximize the yield of soluble insect protein production. Further, the effects of processing steps applied and extraction conditions used on protein and selected techno-functional properties of insect intermediates need to be investigated to predict possible applications of insect-based intermediates and products in the food and feed area. To be able to offer sustainable insect fractions, intermediates, and products to the consumer, it is further important to use sustainable processing technologies and handling along the food chain. For an economic and safe industrial mass production of edible insects, excessive research is required regarding cost-effective rearing methods and post-harvest processing technologies including the development of effective decontamination, modification and storage procedures [167, 168]. As the way in which food is produced will need to change, to be able to cope with the increased demand on natural resources, also the application of innovative technologies should be taken into consideration in order to meet the future requirements.

Insects are often contaminated with pathogenic and non-pathogenic microorganisms [169] and have the potential to cause food spoilage and intoxication or function as vectors transmitting zoonosis and thus, need to be processed and stored properly. For this purpose, it is necessary to develop and employ effective decontamination procedures including thermal and nonthermal techniques in order to ensure food and feed safety. Rumpold *et al.* [170] reviewed traditional decontamination and preparation methods and further evaluated and compared thermal and innovative inactivation methods for the decontamination of meal worm (*Tenebrio molitor*) larvae. They found that indirect plasma treatment was an effective means for the surface decontamination of meal worm larvae, whereas high hydrostatic pressure at 600 MPa and thermal treatments in a water bath at 90 °C in comparison resulted in the highest reduction of the overall microbial count. It is thus concluded that volumetric methods are favorable for the inactivation of the gut microbiota of insects.

Further studies on applying semi-direct plasma on *T. molitor* larvae and flour produced thereof showed that CAPP generated by using a DBD plasma setup was capable of effectively reducing the total viable count (TVC) on the surface of the larvae (Bußler, *unpublished data*). A continuous mass loss was detected upon extended CAPP treatment (Fig. 19), most likely caused by the evaporation of water, since the core temperature of the meal worms rose to 63.3°C during exposure to plasma for 15 min. Taking into account the plasma-induced removal of water, the initial TVC of 2.6 log CFU/g_{DM} on the larvae surface was reduced by 1.75 log cycles within the first 2.5 min of exposure to CAPP. The overall TVC of *Tenebrio* larvae (3.0 log CFU/g_{DM}) was decreased by 1.3 log cycles following 7.5 min of CAPP treatment, whereas prolonged exposure to plasma did not lead to further reduction of the TVC.

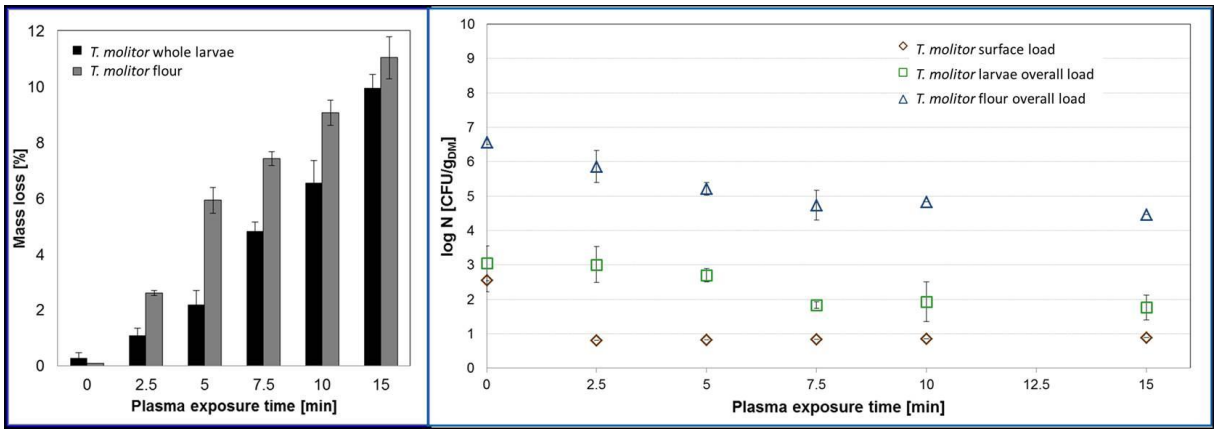

Figure 19: Mass loss of Tenebrio larvae and flour detected following CAPP treatment for up to 15 min (left) and impact of CAPP treatment for up to 15 min on total viable count (TVC) on surface and of whole Tenebrio larvae and on TVC of Tenebrio flour corrected by the respective final dry matter contents (Bußler, unpublished data).

As the CAPP technology is known to be a surface treatment, increasing the surface of the product exposed to plasma-immanent species may lead to an increase in inactivation efficacy. To this end, the application of CAPP to flour produced from *Tenebrio* larvae may offer an advantage with respect to also effectively inactivating the gut microbiota of the larvae (Fig. 19). On *Tenebrio* flour, a TVC of 6.5 log cfu/g_{DM} was detected, which was reduced by 2.1 log cycles with exposure to CAPP for 7.5 min. Besides increasing the decontamination efficacy of

the treatment, exposing the flour to CAPP was further found to be advantageous, as browning reactions, which occurred during CAPP treatment of *Tenebrio* larvae, could be completely prevented. Undesired browning of *Tenebrio* larvae and flour also occurs as a consequence of thermal treatments, as boiling or hot air drying. Therefore, the application of CAPP as a pre-treatment or in combination with other process steps may offer substantial advantages compared to thermal treatments during post-harvest processing of insects (Fig. 20).

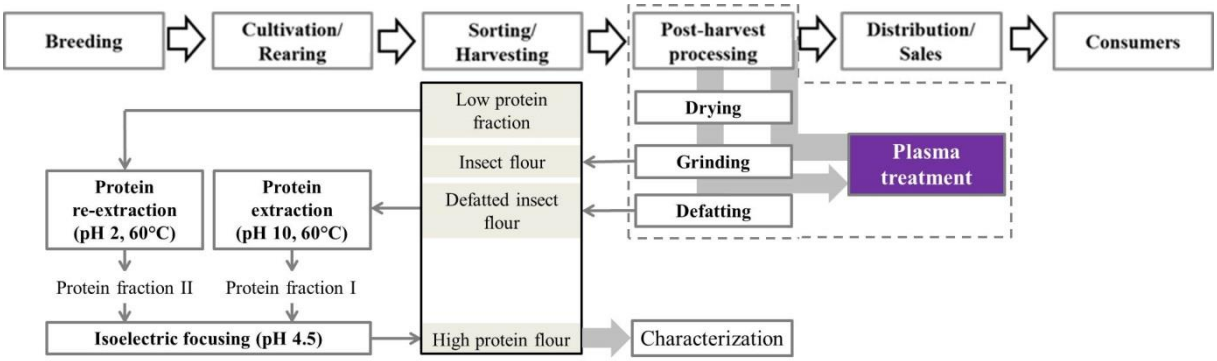

Figure 20: Potential integration of the CAPP technology into the value-added chain of larvae from edible insects.

Investigations on the possible use of the CAPP technology for the decontamination and modification of flour produced from mealworms (*T. molitor*) should be compared to the effects obtained with those induced by a traditional thermal treatment. Besides investigating the plasma and heat induced inactivation of the native microorganism flora special focus must be set on monitoring the process-specific impact on techno-functional and protein properties. In order to gain deeper knowledge on plasma-induced changes in protein solubility, structure and composition, interest should further be directed towards analyzing the contained *Tenebrio* proteins. Proven underlying mechanisms may provide a basis for the targeted use of the CAPP technology as a tool for functionalization and modification of insect-based products.

This part of the thesis aims at investigating the integration of the CAPP technology into the value-added chain of insects by

i. characterizing the raw material in detail including composition, protein- and techno-functional properties, as well as conducting first examinations regarding the general processing and fractionation of insect flours (Section 4.2.1), and

ii. the comparison of effects induced by innovative CAPP processing on *Tenebrio* flour and protein modification and microbial decontamination, which represent key issues in the value-added chain of edible insects, to effects induced by conventional thermal treatment with dry heat (Section 4.2.2).

4.2.1 Recovery and techno-functionality of flours and proteins from two edible insect species: meal worm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae

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Recovery and techno-functionality of flours and proteins from two edible insect species: Meal worm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae

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Abstract

Depending on the species, edible insects are highly nutritious and thus represent a noteworthy alternative food and feed source. The current work investigates the protein extractability and techno-functionality of insect flour fractions recovered from *Tenebrio molitor* and *Hermetia illucens*. *T. molitor* and *H. illucens* flours contained about 20% crude fat and 60% and 36 % crude protein, respectively. Defatting reduced the crude fat content to 2.8% (*T. molitor*) and 8.8% (*H. illucens*) and increased the crude protein content to 68% and 47%, respectively. To isolate proteins from the flours, protein solubility was optimized by varying the pH, the ionic strength, and the extraction temperature of the solvent. All products and by-products accumulated in the protein production process were characterized by composition, selected techno-functional properties, protein solubility, composition and structure as well as their microbial load.

Keywords: Food Science

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1. Introduction

Recently, insects have gained more and more attention in Europe as an underexploited sustainable protein and nutrient source for food and feed. Their potential has been pointed out for example in two FAO publications (Durst et al., 2010; Van Huis et al., 2013) and several reviews (Barroso et al., 2014; Makkar et al., 2014; Nowak et al., 2016; Rumpold and Schlüter, 2013; Sánchez-Muros et al., 2014). A risk profile published by the EFSA (EFSA, 2015) has emphasized the numerous uncertainties and knowledge gaps regarding the use of insects and products thereof as food and feed. In addition, consumer acceptance is a major challenge. In general, western consumers may be reluctant to accept insects as a legitimate protein source because they have never played a substantial role in their food culture. In an exploratory research, 32 Italian consumers, aged 20–35 years, were interviewed in groups on their willingness to eat insect-based food products. It was discovered that this willingness depends on the presented form of the products (Balzan et al., 2016). This was confirmed by a Dutch study on meat replacers (Schösler et al., 2012) where the authors found that the consumer acceptance of insects in food products increased when insects were not visible in modified products indistinguishable from familiar ones. This suggests it is favorable to introduce insects to the human consumer in a masked form as powder, meal or fraction. However, supplementing food products with insect-based hemi-products/ingredients, proteins and fractions requires extensive knowledge on their properties. In case of proteins, these properties include, among others, solubility, amino acid profile, thermal stability and techno-functional properties as water and oil binding, gelling, foaming and emulsifying capacity. Separating extracted protein groups based on their solubility in solvents produces water-soluble and water-insoluble fractions, which can be used for specific applications in the food industry.

Extracting insect proteins for human food products – a process already being carried out – could be a useful way of increasing acceptability among wary consumers. There is little scientific data published on protein extraction from insects. Del Valle et al. (1982) performed a protein extraction from the Mexican fruit fly *Anastrepha ludens* with a maximum protein solubility at pH 10 and subsequent protein precipitation at pH 5. A protein concentrate with a protein content of 65.4% and a protein isolate with a protein content of 86.6% were obtained (based on dry matter, respectively). Investigation of the functional *A. ludens* protein properties resulted in considerably lower foaming and emulsion capacities compared to egg white protein. The solubility of the extracted fly proteins was highest at a pH of 10 (95%) and lowest at the isoelectric point at a pH of 5 (8%).

Yi et al. (2013) investigated the techno-functional properties of proteins from five insect species: *Tenebrio molitor* (larvae), *Zophobas morio* (larvae), *Alphitobius*

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diaperinus (larvae), *Acheta domesticus* (adult) and *Blaptica dubia* (adult). An aqueous protein extraction was performed. The protein purity based on dry matter ranged from 50–75%. It was observed that the insect proteins investigated had the ability to form gels depending on their concentration and on the pH having the potential to be used as gelling agents or texturizers in food (Yi et al., 2013). A comparison of three differently produced protein extracts from aphids using mass spectrometry and gel electrophoresis suggests that the protein extraction methods influence the properties of the extracted protein (Cilia et al., 2009). Mariod et al. (2011) extracted the protein gelatin from the two defatted, dried and ground Sudanese beetles *Aspongubus viduatus* and *Agonoscelis pubescens*. The applicability of the gelatin extracted from insects in comparison to commercially available gelatin as a stabilizer in the ice cream production was investigated and was rated as acceptable by a panel. In addition, there were no significant differences by the general preferences between ice cream produced with insect gelatin and produced with commercial gelatin (Mariod, 2013).

A targeted application of insect-derived ingredients in food formulae is facilitated by insect processing and protein extraction. For an industrial bio-fractionation, established processing chains for the production of high-quality and affordable proteins from traditional protein sources need to be adapted to meet the specific requirements of edible insects as a raw material. In this context and in contrast to the study published by Yi et al. (2013) who recovered soluble insect proteins by a simple aqueous extraction procedure and concluded that research is needed for developing further extraction and purification procedures, and for more detailed insight into functional properties, aim of the present study was to characterize the proteins from *Tenebrio molitor* and *Hermetia illucens* under varying extraction conditions (pH, ionic strength and temperature) and to investigate the composition and properties of recovered insect flour fractions. A further goal and differentiation to published studies was to identify the necessary process stages and extraction parameters required in order to maximize the yield of soluble insect proteins. For these purposes, the intermediate products *T. molitor* flour (T-F) and *H. illucens* flour (H-F), defatted *T. molitor* flour (T-DF) and *H. illucens* flour (H-DF), *T. molitor* high protein fraction (T-HPF) and *T. molitor* low protein fraction (T-LPF) were characterized for their techno-functional, microbial and protein properties. The effects of nonthermal processing steps applied and extraction conditions used on protein and selected techno-functional properties of insect intermediates were investigated to predict their application in food and feed processing.

2. Material and methods

Black soldier fly (*Hermetica illucens*) and yellow mealworm (*T. molitor*) larvae are among the most promising insects for industrial production in the western world. These two species were used in the present work in order to study the applicability

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of a cold wet process as a preparation for the protein extraction on a laboratory scale. The process steps chosen ensure a minimization of thermal effects for the production of insect flours containing proteins in its native form.

2.1. Processing of the *T. molitor* and *H. illucens* larvae

T. molitor larvae, purchased from a local breeder (Futtertier-Shop.de, Eisenhüttenstadt, Germany) and *H. illucens* larvae, purchased from Hermetia Baruth GmbH (Baruth, Germany), served as test material. A schematic depiction of processing and fractionation of the insect larvae is given in Fig. 1. Larvae were separated from frass by sieving, then packaged in freezer bags, subsequently inactivated by freezing and stored at −20 °C.

Non-defatted insect flours were produced by pureeing frozen larvae with distilled water (1:1 w/w) at 4 °C, followed by freezing at −20 °C, freeze drying (Christ Alpha 1–4, Christ Gefriertrocknungsanlagen, Osterode, Germany) and grinding (Clatronic KSW 3307, Clatronic International GmbH, Kempen, Germany).

Removal of fat from the obtained *T. molitor* (T-F) and *H. illucens* (H-F) flours was conducted by a two-step extraction of the fat with hexane. One part of the respective insect flour and five parts of hexane were stirred on a magnet stirrer for 1 h. Following sedimentation of the solids, the hexane-fat-mixture was decanted. The procedure was repeated twice. Residual hexane was removed by evaporation overnight. Subsequent fine grinding of the defatted low-fat fractions using a coffee mill produced defatted *T. molitor* (T-DF) and *H. illucens* (H-DF) flours.

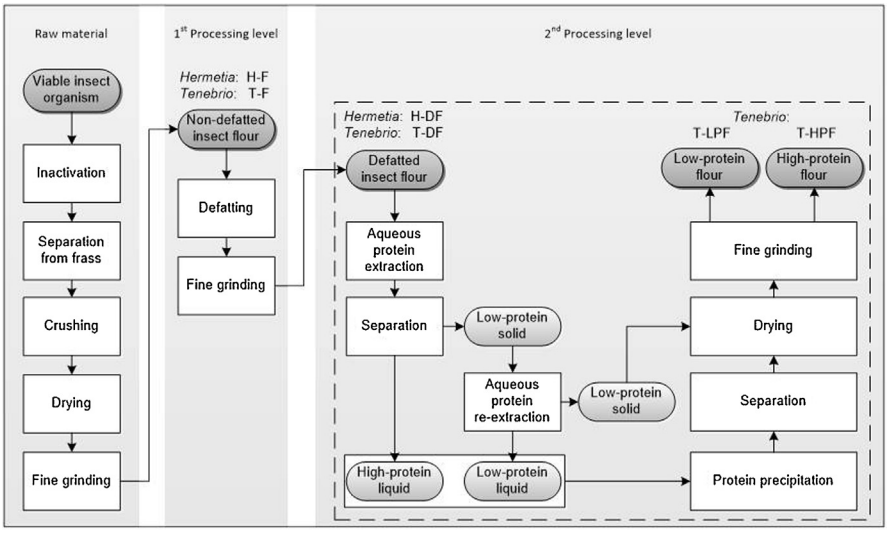

Fig. 1. Schematic representation of processing and fractionation of larvae from *T. molitor* and *H. illucens*.

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A high-protein fraction (T-HPF) was recovered from T-DF by aqueous extraction of the soluble proteins with distilled water (1:25 w/v) adjusted to pH 10 (1 M sodium hydroxide) under stirring (300 rpm) at a constant extraction temperature of 60 °C for 30 min. The recovered extract was centrifuged at 4000 g and 20 °C for 20 min. The clear supernatant was collected and proteins were precipitated by adjusting the pH to 4 (1 M hydrochloric acid). Re-extraction (pH 2, 60 °C, 30 min) and precipitation of the residual proteins from the solids were conducted as previously described. Both protein-rich extracts were centrifuged (4000 g, 20 °C, 20 min). Proteins were frozen (−80 °C), freeze dried, ground and unified. The low-protein fraction (T-LPF) consisted of the insoluble residues recovered during aqueous protein extraction which were unified, freeze dried and fine ground.

2.2. Characterization of quality and techno-functional parameters

2.2.1. Crude protein, crude fat and dry matter content

Crude protein contents (N_{Kjel}, conversion factor 6.25) were determined by the Kjeldahl method (Kjeldatherm Turbosog, Titrino plus 848, Gerhardt Analytical Systems, Königswinter, Germany), according to DIN EN 25663 and as described by the Association of German Agricultural Investigation and Research Institutions (VDLUFA, 1976). Crude fat content of the flour fractions was analyzed using the filter bag (Filterbags XT4, ANKOM Technology, New York, USA) method Am 5-04 (AOCS, 1998; AOCS, 2005). Dry matter contents of the insects and insect flour fractions were determined by oven drying (105 °C, 48 h).

2.2.2. Color measurement

To measure the impact of the different processing steps on the color of the insects and insect derived products, the HunterLab-system was used. As described by Bußler et al., (2015), a Minolta spectrophotometer (CM-2600D, Konica Minolta Inc., Osaka, Japan) was set at illuminant D65, 3 mm aperture, and 0° viewing angle taking L-values (brightness), a-values (green–red axis), and b-values (blue–yellow axis) for nine samples of each product. Following Eq. (1), the change in color (ΔE) was calculated, whereas the indices 0 and p indicate measured values of unprocessed (larvae) and processed insects (flour fractions).

$$\Delta E = \sqrt{(L_0 - L_p)^2 + (a_0 - a_p)^2 + (b_0 - b_p)^2} \quad (1)$$

Following Eqs. (2) and (3), the browning index (BI) was calculated.

$$BI = \frac{[100 \cdot (x - 0.31)]}{0.17} \quad (2)$$

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$$x = \frac{(a + 1.75 \cdot L)}{(5.645 \cdot L + a - 3.012 \cdot b)} \quad (3)$$

2.2.3. Water (WBC) and oil binding capacity (OBC)

To determine the impact of the processing steps on the WBC of the insect flour fractions the method by Smith and Circle (1978a, 1978b), modified by Quinn and Paton (1979) was applied. Therefore each 0.5 g of the respective insect flour fraction was weighted into centrifuge beakers. Samples were stirred (60 s) with 2.5 mL of water using a propeller stirrer and an overhead agitator (Yellowline®, IKA® OST basic, New Jersey, USA). Following a 20 min centrifugation step, (3900 g) the samples were re-weighed after decanting the supernatant and putting the beaker upside-down on filter paper for 60 min. Following Eq. (4), WBC was calculated.

$$WBC\left[\frac{g_{water}}{g_{DM}}\right] = \left(\frac{m_0 - m_1}{m_{0,DM}}\right) \quad (4)$$

Whereby, m_0 is the initial weight of the sample, m_1 is the final weight of the sample and $m_{0,DM}$ is the initial weight of the sample based on dry mass (Bußler et al., 2015; Reinkensmeier et al., 2015).

For the determination of the OBC, the method by Schwenke et al. (1981) was applied. Here, 0.5 g of the respective insect flour fraction was stirred with 2.5 mL of commercial rape seed oil two times for 60 s at 1000 rpm with a five-minute intermission in between. Following centrifugation and re-weighing, OBC was calculated similar to WBC (Eq. (4)).

2.2.4. Emulsifying capacity (EC)

Emulsifying capacity (EC) of the T-DF was tested dependent on the protein concentration and the pH. For this purpose 0.1% protein solutions were prepared at pH 5 and 7 and diluted to final protein concentrations of 0.02, 0.04, 0.06, 0.08 and 0.1%. Each 5 mL of the respective protein solution were put into 50 mL tubes. Rapeseed oil dyed with liquid natural carotene (M = 536.89 g/mol, Carl Roth, Karlsruhe, Germany) was added dropwise using an 20 mL automatic burette (solarus, Hirschmann Laborgeräte, Eberstadt, Germany) under continuous dispersion (9500 rpm, Ultra turrax, IKA, Staufen, Germany). The maximum oil volume emulsified was read off with phase separation. EC was calculated following Eq. (5), whereas v_{oil} was the volume of oil emulsified, v_{ps} was the volume of protein solution used and c_{ps} was the protein concentration of the aqueous phase.

$$EC\left[\frac{mL}{mg}\right] = \frac{v_{oil}[mL]}{v_{ps}[mL] \cdot c_{ps}\left[\frac{mg}{mL}\right]} \quad (5)$$

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2.3. Characterization of insect proteins and protein properties

2.3.1. Protein solubility

For testing the solubility of the contained insect proteins, 0.2 g of the respective insect flour fraction were weighed into a small beaker. A pH dependent extraction of the insect proteins was conducted by adjusting the pH (2 to 12) of the extracts using 1 M hydrochloric acid or 1 M sodium hydroxide. Thereof resulting deviations in the extraction ratio were protocolled and factored into the calculation of the protein concentration. Sodium chloride was used to adjust the ionic strength of distilled water between 0.05 and 10 M. Extraction under varying temperatures was conducted with preheated distilled water while maintaining the respective temperature in a water bath with a built-in shaker. Extraction was carried out under stirring on a rotary shaker (350 rpm) using 5 mL of the previously described solvents. Protein extracts were centrifuged for 10 min (10,000 g, 4 °C) and the clear supernatant was subsequently analyzed.

The Biuret assay (Robinson and Hogden, 1940) was used for quantitative protein analysis. Biuret reagent was prepared as described elsewhere (Bußler et al., 2015; Bußler et al., 2016).

Bovine serum albumin (Fluka, Buchs, Switzerland, c_{Biuret} = 0–10 mg/mL in 2 mg/mL intervals) served as standard and; the assay consisted of 200 µL of the protein extracts and 800 µL of Biuret reagent reacting for 45 min at ambient temperature. The absorption maximum was measured at 540 nm against a blank value (respective solvent) using an UV/Vis spectrophotometer (BioPhotometer plus, Eppendorf, Hamburg, Germany). The amount of soluble insect protein was related to dry matter and crude protein content (Kjeldahl) of the respective insect flour fraction (Bußler et al., 2015).

2.3.2. Fluorescence measurement

Fluorescence emission spectra were measured using a PerkinElmer LS55 fluorescence spectrometer (Rodgau-Jügesheim, Germany) equipped with a pulsed xenon lamp and a red-sensitive photomultiplier (R928) (Bußler et al., 2015) at an excitation wavelength of 280 nm. The fluorescence spectra were scanned in a wavelength range of 300–550 nm placing a cut-off filter at 290 nm in front of the emission monochromator (slit width 5). As described by Bußler et al. (2015), differences in protein concentration and pH of the samples were equalized by dilution with 0.1 M phosphate buffer (pH 7). Measurement was conducted in triplicate using phosphate buffer as the blank.

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2.3.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

For determining the molecular weight distribution of the insect proteins SDS-PAGE according to Laemmli (1970) was used. As described by Reinkensmeier et al. (2015), the pooled samples (n = 3) were mixed in a ratio of 1:10 with sample buffer (0.0125 M Tris buffer at pH 6.8 containing 0.005 M EDTA at pH 6.8–7.0, 1% of sodium dodecyl sulphate, 10% of glycerol, 1% of 2-mercaptoethanol and 0.005% of Bromophenol Blue). Denaturation of the proteins was conducted at 95 °C for 3 min prior to analysis. Vertical electrophoresis equipment (Mini-PROTEAN) from Bio-Rad (Bio-Rad Laboratories GmbH, Munich, Germany) were used to prepare the gels. As standard the PageRulerTM Unstained Broad Range Protein Ladder (Thermo scientific, Vilnius, Lithuania) was used. The band intensity of 5 µl/10 µl of the samples separated in 12% T gels was estimated following staining the gels with Coomassie Brilliant blue and quantification was conducted using analysis Software (Quantity One 1-D, version 4.5.2, Bio-Rad, Milan, Italy).

2.4. Microbial analysis

For determining the impact of insect processing on the overall microbial count of the mealworm flour fractions, 3 g of the respective material and 27 g of 0.1% casein–peptone-solution (CPS) were mixed in a sterile filter stomacher bag and homogenized (Bag Mixer Interscience, St. Nome, France) at speed 8 for 2 min. Following serial dilution of the homogenates with CPS in Rotilabo®-microtest plates (96er U-profile, Roth, Germany), 50 µl of each dilution were spread on plate count agar. Following incubation at 30 °C for 72 h the number of colony forming units per g on a dry matter basis (CFU/g_{DM}) was determined with a detection limit of plate count analyses of 200 CFU/g_{DM}. All analyses were carried out at least in triplicates.

2.5. Statistical analysis

Extractions and following analytical steps each were conducted in triplicate (n = 9), total viable counts were determined from three independent samples preparing homogenates in duplicate (n = 6). All data were statistically analyzed (ANOVA) with StatisticaTM for WindowsTM (version 9.0, Statsoft Inc., Tulsa, OK, USA) determining significant differences between means by Turkey's HSD test ($p < 0.05$). The mean variability of data was indicated by the standard deviation in the figures.

3. Results and discussion

Processing of *T. molitor* and *H. illucens* larvae affected composition, appearance, microbial load as well as techno-functional and protein properties of the recovered

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insect products. The processability of larvae from both insect species was limited by their high fat contents. Direct processing of frozen or dried larvae into flour was found to be non-practicable due to the thermally induced melting of the contained fat during grinding. In order to avoid any thermal impact during preparation of the flour fractions and to maintain the native properties of the contained proteins, the temperature was kept below 20 °C while performing the procedure described above. Initial experiments indicated that the process route applied was less suitable for achieving the desired process objectives regarding the necessary product properties required for detailed analysis in case of *H. illucens*. In particular the non-removable fat limited the processability and analysis of the respective flour fractions. For this reason detailed characterization providing reliable results was limited to *T. molitor* flour fractions.

3.1. Impact of extraction process on yield, composition and color of flour fractions from *T. molitor* and *H. illucens*

Composition of *T. molitor and H. illucens* larvae is shown in Table 1. Containing comparable amounts of water and fat, the protein content in *T. molitor* larvae was 22.1% higher compared to *H. illucens* larvae. *T. molitor* larvae had a dry matter content of 34.9% and contained 53.8% of crude protein and 20.0% of crude fat on a dry basis. Dependent on the growth stage of *T. molitor* larvae, Ghaly and Alkoaik (2009) reported dry matter contents ranging from 38.5 to 41.9%, crude protein contents between 24.3 and 27.6% and crude fat contents from 12.0 to 12.5% on a fresh weight basis, respectively. The results were comparable to moisture, crude

Table 1. Means (±sd) of yield, dry matter (DM), crude protein (CP), crude fat content (CF), browning indices (BI), and change in color (ΔE) of larvae from *T. molitor* and *H. illucens* and different flour fractions produced from it as well as total viable count (TVC) of the *Tenebrio* flour fractions. Different letters indicate significant ($p < 0.05$) differences between means.

Flour fraction	Yield [%]	DM [g/g]	CP [g/g_{DM}]	CF [g/g_{DM}]	BI [−]	ΔE [−]	TVC [log CFU/g]
Tenebrio molitor							
Larvae	–	34.9^{a} (±1.2)	53.8^{a} (±1.0)	20.0^{a} (±1.0)	52.8^{a} (±8.7)	–	8.1^{a} (±0.1)
T-F	96	83.8^{b} (±0.9)	57.8^{b} (±1.2)	19.1^{a} (±1.3)	26.6^{b} (±6.6)	5.4^{a} (±0.5)	7.9^{a} (±0.2)
T-DF	83	87.5^{c} (±0.4)	64.6^{c} (±0.3)	2.8^{b} (±0.3)	37.0^{c} (±5.7)	6.7^{b} (±0.3)	7.0^{b} (±0.5)
T-HPF	22	96.5^{d} (±0.2)	68.2^{d} (±0.3)	0.4^{c} (±0.0)	19.0^{d} (±4.2)	15.0^{c} (±0.6)	4.3^{c} (±0.2)
T-LPF	21	98.9^{e} (±0.1)	11.2^{e} (±0.2)	2.2^{d} (±0.3)	53.7^{e} (±5.6)	9.1^{d} (±0.2)	6.2^{d} (±0.1)
Hermetia illucens							
Larvae	–	30.0^{a} (±1.2)	31.7^{a} (±0.5)	21.1^{a} (±0.7)	27.8^{a} (± 7.1)	–	–
H-F	82	84.1^{b} (±0.7)	34.7^{b} (±0.2)	20.0^{b} (±0.8)	19.2^{b} (± 5.2)	10.2^{a} (± 0.4)	–
H-DF	73	87.0^{c} (±0.3)	44.9^{c} (±1.4)	8.8^{c} (±0.1)	52.8^{c} (± 8.7)	7.7^{b} (± 0.6)	–

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protein and crude fat contents reported by Yi et al. (2013). On a dry basis, the protein and fat contents of the yellow mealworms were in the ranges of 63.3–68.9 and 29.8–31.2%, respectively. With a moisture content of 30%, *H. illucens* larvae contained 31.7% crude protein and 21.1% crude fat on a dry basis. Compared to findings reported in the literature, protein and fat content were lower. Booram et al. (1976) reported that the *H. illucens* larvae consisted of 42% crude protein and 35% crude fat whereas Kroeckel et al. (2012) found 54.1 ± 1.1% crude protein and 13.4 ± 0.7% crude fat, respectively. The dry matter contents of the different flour fractions increased with increasing degree of processing. The crude protein content of the *T. molitor* larvae was increased by 4%, 10.8% and 14.4% in the T-F, the T-DF and the T-HPF. The T-LPF had a residual protein content of 11.2%. Production of H-F and H-DF increased the crude protein content by 3.0 and 13.2%, respectively. Here, the initial fat content of the larvae was reduced by 1.1 and 12.3%. Although insects flours contained nearly the same quantity of fat, defatting of the H-F was less effective.

Processing of the insect larvae further affected visual appearance of the flour fractions produced. Changes in color are summarized in Table 1. In general the *T. molitor* larvae was darker compared to *H. illucens*. This is confirmed by the higher browning index of the whole *T. molitor* larvae. Grinding of the mealworms to non-defatted flour slightly increased the browning index and induced a change in color compared to the unprocessed larvae, whereas the non-defatted flour from *H. illucens* larvae appeared lighter. Defatting with hexane led to a less brownish color of the flours produced from both insect species. Protein extraction from defatted T-DF, subsequent precipitation, drying and grinding produced the dark brown colored T-HPF and the lighter T-LPF. The color of the different insect flour fractions seems to be related to the protein content, whereas the browning index was found to be dependent on the fat content.

Following aqueous extraction of *T. molitor* proteins in the pH range from 2 to 12, the protein extracts had a light yellow color in the acidic pH range (2–6), a light brown color at pH 7, and a dark brown color in the alkaline pH range (8–12). In addition, the color of the residue fraction was similar to that of the supernatant fractions. This visual observation indicated that chemical reactions took place during protein extraction under varying conditions. Preliminary experiments showed that color formation was most likely due to enzymatic browning reactions and also depended on the protein concentration of the respective extract.

3.2. Impact on techno-functional properties

Processing of the *T. molitor* and *H. illucens* larvae affected techno-functional properties of the flour fractions produced. WBC and OBC of the flour fractions recovered during protein isolation from *T. molitor* are depicted in Fig. 2. No

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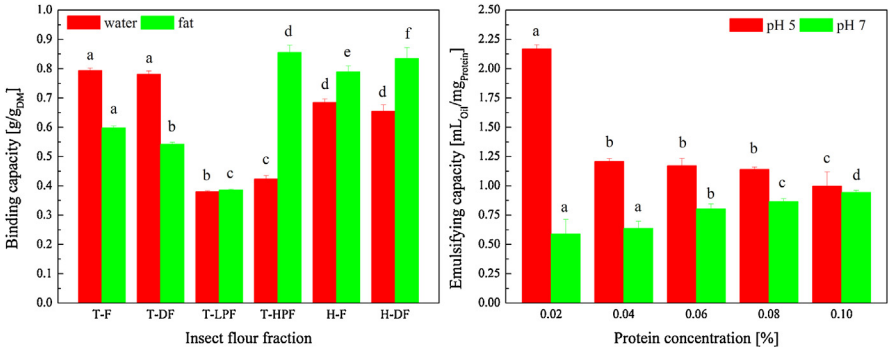

Fig. 2. Water and fat binding capacities (left) on a dry basis [g/g_{DM}] of the *T. molitor* flour fractions (T-F = *T. molitor* flour, T-DF = defatted *T. molitor* flour, T-LPF = low-protein fraction, T-HPF = high-protein fraction) and emulsifying capacities [mL_{Oil}/$mg_{Protein}$] of defined protein solutions prepared from defatted *T. molitor* flour (right) in dependency of the protein concentration (0.02–0.1%) and the pH (4 and 10) of the aqueous phase. Different letters indicate significant ($p < 0.05$) differences between means.

significant impact of the defatting step on WBC was observed whereas WBC of the T-HPF and T-LPF were significantly decreased by 0.41 g/g_{DM} and 0.37 g/g_{DM}, respectively. A slight decrease in OBC by 0.05 g/g_{DM} was observed by defatting of the *T. molitor* flour. The OBC of T-LPF was significantly decreased by 0.21 g/g_{DM} whereas it was increased by 0.26 g/g_{DM} regarding the T-HPF.

In case of *H. illucens*, defatting of the flour did also not result in significant changes in WBC, whereas OBC was marginally increased by 0.05 g/g_{DM}. Up to now, no comparable research is reported in literature. Yi et al. (2013) investigated foamability, foam stability and gelation of soluble proteins from five insect species and found poor foaming capacities at pH 3, 5, 7, and 10, but the formation of gels at a concentration of 30% w/v with gelation temperature ranging from about 51 to 63 °C for all insect species at pH 7. With regard to food applications, WBC is related with the ability to retain water against gravity, and includes bound water, hydrodynamic water, capillary water and physically entrapped water. The amount of water associated to proteins is closely linked to its amino acids profile, increases with the number of charged residues (Kuntz and Kauzmann, 1974) and strongly depends on protein conformation, hydrophobicity, pH, temperature, ionic strength and protein concentration (Damodaran, 1997).

Emulsion capacity (EC) denotes the maximum amount of oil that can be emulsified under specified conditions by a unit weight of the protein. In this study, the EC of protein solutions prepared from T-DF was investigated under varying protein concentration and pH of the aqueous phase (Fig. 2) and was found to be highly dependent on these two parameters. Emulsification at pH 5 led to an EC of 2.35 mL_{Oil}/$mg_{Protein}$. It decreased with increasing protein concentration. At pH 7 the EC of a 0.02 mg/mL protein solution was significantly lower (0.64 mL_{Oil}/$mg_{Protein}$)

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and increased to 0.87. Emulsification characteristics of proteins for instance are affected by their surface hydrophobicity as it influences the ability for the protein to adsorb to the oil side of the interface. Greater disintegration typically leads to higher emulsion capacities (Kim et al., 2005). Properties of adsorbed layers at oil-water interfaces has been explained on the basis of a 'molten globule state' concept of globular proteins such as α-lactalbumin and β-lactoglobulin. It was found that partially denatured state of globular proteins that retains the secondary structure but not the tertiary structure of the native protein (i.e. increased flexibility of molecules) explains their behavior at an oil-water interface. Some level of partial protein denaturation or a change in molecular charge distribution may lead to the exposure of buried hydrophobic amino acids to the surface. In this case, proteins re-align at the interface in order to position their surface hydrophobic amino acids within the oil phase and hydrophilic amino acids within the aqueous phase. Further, surface charge of the protein influences protein solubility within the aqueous phase. High electrostatic repulsion between oil droplets tends to lead to greater emulsion stability, whereas under pH conditions close to the protein's isoelectric point (or high ionic strength) droplet flocculation/aggregation may dominate eventually leading to coalescence and instability. Regarding the EC of *T. molitor* protein this could be an explanation for the higher EC at pH 5 at lower protein concentrations. However, the decreased EC at pH 7 in comparison cannot be explained and needs to be further investigated.

3.3. Protein solubility and structure

Solubility of the proteins contained in both insect species was found to be highly dependent on the pH during extraction process (Fig. 3 and Fig. 4, top). The insect proteins had their isoelectric point (pI) in the region around pH 4. Regarding T-F, proteins showed highest solubility in the alkaline region at pH 10. Except for this pH, defatting of the flour led to increased protein yields over the entire pH range but especially in the acidic region at pH 2 and 3. Protein yield of H-F was significantly lower. H-F proteins had a higher solubility in the alkaline region at pH 12 and also in the acidic region at pH 2. Hexane extraction of the fat did not lead to increased yields in soluble protein; on the contrary, protein solubility was even decreased at low pH values.

For all insect flour fractions the pI was found to be in the region of pH 4. For a great number of proteins, their pI values are in the range of 3.5 and 6.5. At extreme acidic or basic pH values, the protein may unfold exposing more hydrophobic groups. This can be documented by the exposition of the hydrophobic tryptophan residues as measured by the increased fluorescence intensity (Fig. 3 and Fig. 4, bottom). Some insect proteins were reported to have a pI of about 5. For instance, the pI of proteins from silkworm (*Bombyx mori*) and spider (*Nephila edulis*) were found to be in the region between pH 4.37–5.05, and 6.47, respectively (Foo

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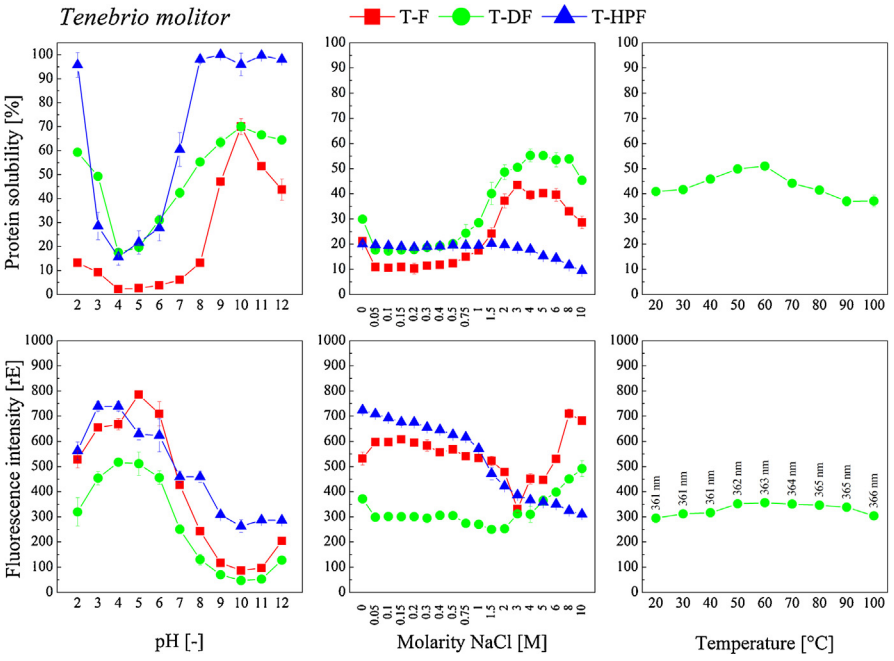

Fig. 3. Soluble portion (top) and fluorescence intensity (bottom) of proteins extracted from *T. molitor* flour fractions (T-F: *T. molitor* flour, T-DF: defatted *T. molitor* flour, and T-HPF: protein fraction) in dependency of the solvent at different pH (2 to 12, left), ionic strength (0 to 10 M NaCl, center) and temperature (20 to 100 °C, right) during extraction. Protein solubility [%] is presented in relation to the total protein content analyzed via Kjeldahl method. Protein concentrations of the extracts were equalized prior to fluorescence measurement.

et al., 2006). Here, for both insect species the solubility of proteins extracted from the non-defatted flours was slightly reduced by increasing the ionic strength from 0 to 0.4 M (Fig. 3 and Fig. 4, top). Further increasing the molarity of NaCl to 4 and 3 maximized the protein solubility to 55 (T-F) and 70% (H-F), respectively. With regard to the defatted insect flours, solubility curves were similar. Maximum protein solubility of 43% for both insect species was reached at a NaCl molarity of 3 in case of T-DF and of 6 in case of H-DF. In general, defatting led to a reduction in protein solubility. The solubility curve of the T-HPF significantly differed from those obtained for the other flour fractions. For up to a NaCl molarity of 3, no relevant impact on protein solubility was detected, whereas it decreased with further increasing NaCl molarity to 10. Consequently, increasing the ionic strength of the solvent affects the insect protein solubility, but an increase can only be achieved at high salt concentrations.

Increasing the temperature (Fig. 3 and Fig. 4, top) during protein extraction from 20 to 60 °C significantly increased the protein yields by 20 (T-DF) and 10% (H-DF). In general, protein solubility is increased at temperatures between 50 and 60 °C. In case of the insect proteins, elevated extraction temperatures increased their solubility. This may be attributed to weakened interactions between the

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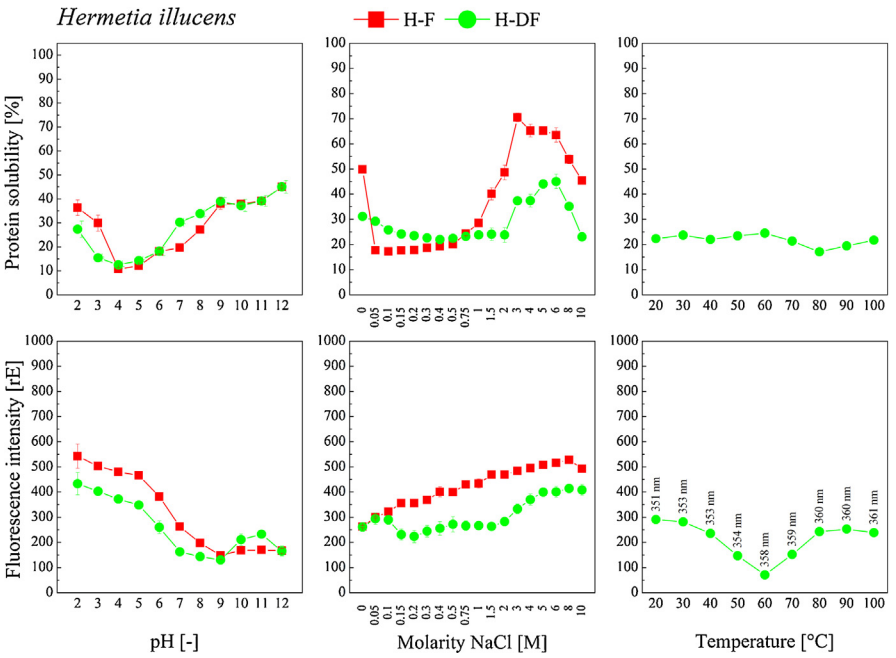

Fig. 4. Soluble portion (top) and fluorescence intensity (bottom) of proteins extracted from *H. illucens* flour fractions (H-F: *H. illucens* flour, and H-DF: defatted *H. illucens* flour) in dependency of the solvent pH (2 to 12, left), ionic strength (0 to 10 M NaCl, center) and temperature (20 to 100 °C, right) during extraction. Protein solubility [%] is presented in relation to the total protein content analyzed via Kjeldahl method. Protein concentrations of the extracts were equalized prior to fluorescence measurement.

proteins and other components as for instance fat. In all of the trials, the presence of non-protein impurities needs to be taken into consideration which may limit protein solubility as proteins may form complexes with lipids or nucleic acids that prevent their full solubilization.

Excited at a wavelength of 280nm, tryptophan emits light in the region between 300 and 350 nm- Changes in tryptophan fluorescence can indicate changes of the conformation and three-dimensional structure of proteins as well as the exposure of the hydrophobic amino acid residues (Gießauf et al., 1995; Vivian and Callis, 2001). In this study, fluorescence spectra of the protein extracts recovered from the insect flour fractions were analyzed. Differences in protein fluorescence maxima (and quantum yields) are most likely caused by various ratios of two or more discrete classes of tryptophan residues contained in proteins on the one hand. On the other hand, the extracted proteins have to be considered as a mixture of several protein components. Following Konev (1967), tryptophyls inside the protein in a low-polar hydrophobic microenvironment are characterized by a short wavelength position of the fluorescent maximum (λ = 331 nm), while tryptophyls on the surface of a protein in a high-polar aqueous microenvironment are characterized by a large Stokes shift (λ = 350 nm). Burstein et al. (1973) also reported that

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tryptophan residues located at the surface of proteins emit light in higher wavelength regions than those located in the core of proteins. Regarding the recorded insect protein fluorescence spectra (Fig. 3 and Fig. 4, bottom), no fluorescence maximum in the region around 330 nm could be detected. All of the *T. molitor* protein extracts were characterized by a fluorescence maximum at around 350 nm whereas it was found at 360 nm in case of *H. illucens* proteins.

Aromatic tryptophan residues are often located in the hydrophobic core of proteins, at the interface between two protein domains/subdomains, or at the subunit interface in oligomeric protein systems and become more exposed to solvent upon disruption of the protein's tertiary or quaternary structure (Bußler et al., 2015). Exposure of tryptophan surroundings to a more polar environment can be one of the reasons for the observed losses of fluorescence emission. The results of the fluorescence measurements suggest the occurrence of structural changes of the insect proteins under varying extraction conditions. In case of varying the pH during extraction, the increase of tryptophan fluorescence intensity with decreasing solubility of *T. molitor* and *H. illucens* proteins is most likely caused by the exposure of hydrophobic residues from the core to the environment of the protein (Fig. 3 and Fig. 4). The two types of binding influenced by pH changes are salt bridges and hydrogen bonding. Whereas an increase in pH leads to the formation of a neutral $-NH_2$ group from $-NH_3^+$ ions, a decrease in pH forms neutral $-COOH$ groups from $-COO^-$ ions. In both cases the ionic attraction is eliminated, and the protein molecule unfolds explaining the different fluorescence emission spectra. In case of *H. illucens* protein extracts, a different behavior was observed. Here, a decrease in protein solubility was not accompanied by an increase in fluorescence emission intensity. There are several effects that need to be taken into consideration. Osysko and Muíño (2011) reported measurements of fluorescence quantum yields of tryptophan, tryptophanylaspartate and tryptophanylarginine in aqueous solutions over a wide range of pH, aiming to test the excitation of quenching in tryptophan caused by energy loss due to an electron transfer from the aromatic system of tryptophan to one of the amides in the protein backbone. Low pH conditions result in a net positive charge for the terminal amine, whereas high pH conditions lead to a net negative charge for the terminal carboxyl. Consequently, increasing (decreasing) electron transfer rates and low (high) quantum yields is to be expected, as a low pH will enhance the probability of electron transfer and thus cause a lower quantum yield, whereas a high pH will decrease the probability of electron transfer resulting in larger quantum yields. We observed that high pH results in a high quantum yield which may be caused by the negatively charged carboxyl inducing very low efficiency for the electron transfer. In turn, low pH results in a low quantum yield.

Varying the extraction temperature induced comparable results regarding the relation between protein solubility and fluorescence intensity. Here again, the

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increase in *T. molitor* protein solubility was accompanied by a decrease in fluorescence emission intensity (Fig. 3) whereas this correlation was not observed in case of *H. illucens* protein (Fig. 4). But the maximum emission wavelength shifted to higher values with increasing temperature. This shift was more pronounced in *T. molitor* protein extracts (from 351 to 361 nm) and less in *H. illucens* protein extracts (from 361 to 366 nm).

3.4. Protein composition

Separation of the *T. molitor* and *H. illucens* proteins via SDS-PAGE using 12% T gels resulted in wide ranges of protein bands (Fig. 5A to D). Quantitative evaluation regarding the protein solubility confirms the results obtained by the Biuret assay depicted in Fig. 3 and Fig. 4. In protein extracts recovered from defatted H-F, two major bands characterized by molecular weights of 14.3 kDa and 80.5 kDa, were dominant (Fig. 5A). At pH 7 the protein extract was composed of 75.9% high molecular weight (HMW) fraction and 24.1% low molecular weight (LMW) fraction. Decreasing the pH to 2 led to a gradual increase in the LMW fraction for up to 71.5% whereas increasing the pH to 12 increased it to 98.9%. It is probable that defatting with hexane in combination with extreme pH conditions during protein extraction led to a partial or almost complete proteolysis of the 80.5 kDa band to 14.3 kDa or even lower.

With regard to *T. molitor* flour fractions, ten major groups of protein bands could be distinguished (Fig. 5B to D), namely bands 13–15 kDa, 15–18 kDa, 18–21 kDa, 21–24 kDa, 24–29 kDa, 30–39 kDa, 40–48 kDa, 48–65 kDa, 67–117 kDa and 128–250 kDa. The percentage distribution of the protein bands in the aqueous extracts varied upon flour fraction used and extraction conditions applied. As already described by Bußler et al. (2016), the bands observed in the range between 14 and 32 kDa could possibly originate from cuticle proteins with molecular weights predominantly between 14 and 30 kDa (Andersen et al., 1995) or chymotrypsin-like proteinase (24 kDa) (Elpidina et al., 2005), whereas the bands observed ranging from 32 to 95 kDa could possibly originate from enzymes and other proteins, e.g. melanization-inhibiting protein (43 kDa), β-glycosidase (59 kDa), trypsin-like proteinases (59 kDa), and melanization-engaging types of protein (85 kDa) (Cho et al., 1999; Ferreira et al., 2001; Prabhakar et al., 2007; Zhao et al., 2005). The bands with molecular weight >95 kDa could possibly be linked to vitellogenin-like protein with a molecular weight of 160 kDa (Lee et al., 2000). With respect to the protein fractions extracted from T-F mainly the proportions of high- and low-molecular fractions were affected by alteration of the solvent pH. Protein fractions characterized by high molecular weights in the range of 67–250 kDa were found to dominate the protein extracts at pH 2 and 3, as they accounted for almost 30% of the total soluble proteins. Adjusting the pH to 2 and 3 increased the solubility of protein fractions in the range between 40 and 250 kDa

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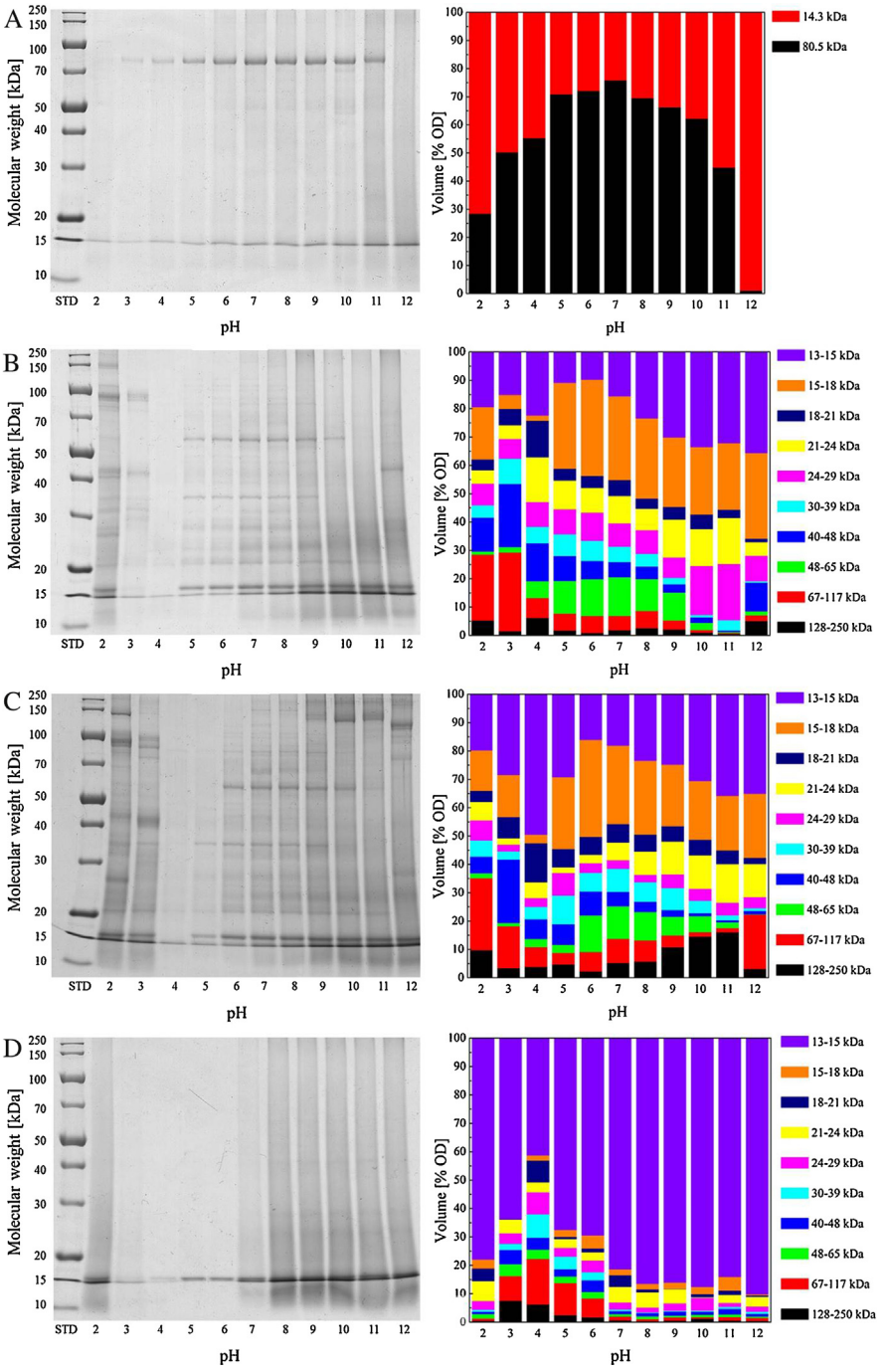

Fig. 5. Electrophoretic separation (left) and relative composition (right) of soluble *T. molitor* and *H. illucens* protein fractions (H-DF: defatted *H. illucens* flour (A), T-F: *T. molitor* flour (B), T-DF: defatted *T. molitor* flour (C), and T-HPF: *T. molitor* high protein fraction (D)) depending on the pH value of the extraction solution, n = 3.

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whereas the amount of the 48–65 kDa and 13–18 kDa fractions increased with pH values from 5 to 8. Furthermore the proportion of LMW fractions in the range of 13–29 kDa increased with alkalization of the solvent pH to 12. Defatting of the T-F significantly affected the protein composition over the entire pH range (Fig. 5C). The percentage proportion of LMW fractions ranging from 13 to 18 kDa was slightly decreased by 4% at pH 2 whereas it was more than doubled (increase from 20 to 43% and from 24.5 to 52.5%) at pH 3 and 4, respectively. In the pH range from 5 to 12 the amount of this protein fraction varied between 41 and 66% prior to and between 48 and 58% following defatting with hexane. Except for the extracts recovered at pH 3 and 4, the proportion of the HMW fraction with a molecular weight in the range between 67 and 250 kDa was higher in the extracts recovered from T-DF. This may be attributed to protein agglomerates formed. The relatively high amounts of the protein fraction with molecular weights ranging from 24 to 29 kDa which were between 17.3 and 19.8% at pH 10 and 11 in the T-F were reduced to 4.2 to 4.5% upon defatting of the flour.

Isolation of the *T. molitor* proteins from T-DF at pH 10 and 2 completely changed the protein composition over the entire pH range (Fig. 5D). The LMW fractions with molecular weights from 13 to 15 kDa accounted for the largest share of soluble *T. molitor* proteins. At pH 12 90.1% of the proteins were found to have a molecular weight in this range. At pH 2 and between pH 7 and 10 the percentage proportion ranged from 77.9% to 86.5% and was reduced at pH 3 (63.7%), 5 (67.5%) and 6 (68.4%). In the pH range of lowest protein solubility (pH 4) the percentage proportion amounted to 41.2%. However, the proportion of HMW fractions (67–250 kDa), which was infinitesimally low at pH 2 due to acid hydrolysis and in the pH range between 7 and 12, amounted to 22.3%. Furthermore, this protein fraction was contained in relevant amounts at pH 3 (16.2%), 5 (13.8%) and 6 (8.4%). The high amount of LMW fractions may be attributed to the proteolytic degradation of proteins during isolation which may be triggered by intrinsic enzymes of the mealworms or of microorganisms.

3.5. Microbial safety

The *T. molitor* larvae were highly contaminated with microorganisms (Table 1). Pureeing, freeze-drying and grinding of the larvae at low temperatures in order to produce the T-F led to an insignificant log-reduction of the total viable count (TVC) by 0.1 (±0.1). Hexane extraction of the fat significantly reduced the TVC by 1.1 (±0.5) log cycles. The most effective process step in decreasing the microbial load was the preparation of the T-HPF via aqueous extraction at pH 10 and 2, precipitation of the soluble proteins at pH 4, freeze drying and grinding. Compared to whole *T. molitor* larvae, the TVC of the T-HPF was significantly

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reduced by 3.8 (±0.0) log cycles. Regarding the low-protein fraction, the TVC was found to be quite high with a log-reduction of 1.9 (±0.0) compared to the raw material. As expected, the process applied is not appropriate for the production of microbially safe incest flour fractions. Extreme pH conditions applied did apparently inactivate microorganisms, only less effectively. For the production of microbial safe insect flours fractions, the application of effective inactivation processes as reported by Rumpold et al. (2014) will be necessary and needs to be studied extensively.

4. Conclusion

The results of this study indicate that edible insects as *T. molitor* and *H. illucens* can be utilized to prepare protein-rich intermediates to be used in the production of food and feed. Despite the solubility characteristics, which were shown to be specific for the insect species used, required processing parameters seem to be similar to those of plant proteins enabling the usage of traditional methods on protein processing. In case of *T. molitor*, T-F was found to be highly soluble at alkaline pH values. The proteins contained in T-DF and T-HPF were also highly soluble at pH 2. Defatting (T-DF) and isoelectric precipitation (T-HPF) increased the concentration of *T. molitor* proteins by 11 and 15%, respectively.

These results further indicate that it is advisable to prepare fat-reduced and protein enriched fractions such as T-DF and H-DF. Further research is required to address bioavailability issues especially with regard to the distribution of amino acid profiles and bioavailability of essential amino acids. The results further indicate that the techno-functional properties can be effectively manipulated, but further research is needed to identify specific tools for tailoring them. It appears that protein composition of the samples may influence the functional properties. Finally, the potential of by-products, as for instance T-LPF and the extracted insect fat providing further options for functionalized added-value products needs to be taken into consideration. Alternative methods could be used for fat removal, thereby omitting environmentally unfriendly use of organic solvents.

Also with respect to microbial safety issues, a combination of several physical separation methods or the application of different thermal treatments may be more suitable techniques for preparing protein rich intermediates rather than extensive isolation procedures. The results obtained in this study clearly underline the importance of tailored process design, especially of the defatting step, when exploiting insects as an alternative protein source and therefore represent an important step towards the development of sustainable and microbiologically safe rearing, harvest and post-harvest processing technologies as well as protein recovery procedures to ensure high food and feed quality.

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Declarations

Author contribution statement

Sara Bußler: Conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; wrote the paper.

Birgit Rumpold: Conceived and designed the experiments; analyzed and interpreted the data; wrote the paper.

Elisabeth Jander, Harshadrai M. Rawel: Performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data.

Oliver Schlueter: Conceived and designed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

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Additional information

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4.2.2 Cold atmospheric pressure plasma processing of insect flour from *Tenebrio molitor*: Impact on microbial load and quality attributes in comparison to dry heat treatment

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Cold atmospheric pressure plasma processing of insect flour from *Tenebrio molitor*: Impact on microbial load and quality attributes in comparison to dry heat treatment

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ABSTRACT

In this study, the applicability of semi-direct cold atmospheric pressure plasma (CAPP) during postharvest processing of *Tenebrio molitor* flour is investigated. Besides analyzing the decontamination efficacy, plasma-induced impact on techno-functionality, protein solubility, composition and structure was determined and compared to heat induced effects.

Following CAPP treatment, the total microbial load of the *Tenebrio* flour of 7.72 $\log_{10}$ cfu/g was reduced to 7.10 (1 min), 6.72 (2.5 min), 5.79 (5 min), 5.19 (7.5 min), 5.21 (10 min) and 4.73 (15 min) $\log_{10}$ cfu/g. With increasing exposure to CAPP, protein solubility at pH 4 almost linearly decreased to a minimum of 54%. Water binding capacity decreased from 0.79 to 0.64 g_{water}/g whereas oil binding capacity increased from 0.59 to 0.66 g_{oil}/g. Gel electrophoresis revealed a decrease of all protein fractions at pH 4 whereas at pH 10 the band pattern significantly shifted to protein fractions with higher molecular weights.

Industrial relevance: Edible insects are rich in valuable protein, fat, fibre, minerals and micronutrients. Although a wide range of species represent a valuable alternative protein source that could contribute to food and feed security, they are industrially hardly exploited. The tailored application of proper processing technologies could lead to novel insect-based high-protein food and feed products with unique functional properties supporting the increase in acceptability among potential consumers. Current research concentrates on developing processing chains including innovative nonthermal approaches. Cold atmospheric pressure plasma (CAPP) has gained attention as an effective technology for the decontamination and modification of fresh and dry agricultural products. In the postharvest chain of edible insects, the application of CAPP could contribute to the development of safe and high-quality insect-based products in the food and feed sector.

1. Introduction

The expanding population is expected to grow to >9.7 billion by 2050 according to the United Nations (United Nations, 2015). As the demand for food will increase dramatically within the forthcoming decades, alternative and sustainable sources of highly nutritious and sustainable food in combination with innovative processing techniques are required.

Edible insects are highly nutritious and can contain high amounts of fat, protein, vitamin, fibre and minerals, thus representing an interesting to date underexploited food source. Existing and new processing pathways need to be adapted or developed in order to produce insect-based safe food ingredients of a high quality, which could be incorporated into numerous consumer items, such as meat substitutes and protein-fortified dry products, including cereals, bars, and snack foods. Whole edible insects represent a traditional food in many parts of the world and are eaten by approx. 2 billion people worldwide (van Huis, 2013). Besides the foreseen development of an effective insect fractionation into a protein, a fat and a chitin rich fraction (Bußler, Rumpold, Jander, Rawel, & Schlüter, 2016), the production of safe and readily processable insect-based intermediates and products represents a wise strategical step towards the industrial use of insects in the food and feed sector. Therefore, it is important to use sustainable processing technologies and handling along the food chain. For an economic and safe industrial mass production of edible insects excessive research is required regarding cost-effective rearing methods and post-harvest processing technologies including the development of effective decontamination, modification and storage procedures (Rumpold & Schlüter, 2013).

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In recent years, significant research effort has been focused on developing and evaluating a multitude of novel nonthermal food technologies with the goal to avoid undesirable effects generated when conventional thermal processes are applied to food matrices such as loss in vitamins and "freshness", undesirable changes in color, texture and taste and protein denaturation. Thereby the research work is primarily motivated by consumer demands for high quality and minimally processed food, whilst ensuring microbiological and chemical safety. In the field of insect processing, too, the application of nonthermal technologies could offer enormous benefits compared to using conventional traditional procedures.

Cold atmospheric pressure plasma (CAPP) also qualifies as a new discipline in food processing. As the CAPP technology was found to be capable of effectively inactivating a wide range of microorganisms including spores and viruses (Baier et al., 2014; Birmingham, 2004; Surowsky, Fröhling, Gottschalk, Schlüter, & Knorr, 2014) it has been considered as an emerging nonthermal technology for the improvement of food safety. Although CAPP offers a promising technology in the different fields of food and feed processing, presently, the only commercial application of CAPP technology in food industries is limited to polymer processing used for food packaging applications (Pankaj, Bueno-Ferrer, Misra, Milosavljević, O'Donnell, Bourke, et al., 2014). It is well-known that CAPP also modifies the structure of materials in the micro- to nanometer range (Attri & Choi, 2013; Pankaj, Bueno-Ferrer, Misra, Milosavljević, O'Donnell, Bourke, et al., 2014; Pankaj et al., 2014b) and researchers found that, similar to the plasma application in material science, CAPP is capable of modifying wet and dry surfaces of agricultural and food products (Grzegorzewski, Rohn, Kroh, Geyer, & Schlüter, 2010; Khanal, Knoche, Bußler, & Schlüter, 2014). Up to now, the unique feature is only used in the non-food sector. Using and transferring knowledge from those research fields and industrial branches to food science and technology may offer an innovative approach for the targeted surface modification und functionalization of powdery and bulky food and feed materials.

Main objective of this study was to investigate the possible use of the CAPP technology for the decontamination and modification of flour produced from mealworms (*Tenebrio molitor*) and to compare the effects obtained with those induced by a traditional thermal treatment. For this purpose a dielectric barrier discharge (DBD) setup with air as the working gas was used as it is applicable for the treatment of larger goods, especially in solid dry powder or granular form. Operation in air reduces the costs when compared to the use of noble gases and the DBD system is a promising choice in order to adopt the CAPP technology for food industry. Besides investigating the plasma and heat induced inactivation of the native microorganism flora, special focus was set on monitoring the process-specific impact on quality, techno-functional and protein properties which will very likely provide specific application possibilities of insect-based intermediates and products. Furthermore, interest was also directed towards analyzing the contained *Tenebrio* proteins in order to gain deeper knowledge of plasma-induced changes in protein solubility, structure and composition which may provide a base for the targeted use of the CAPP technology as a tool for functionalization and modification of insect-based products.

2. Material and methods

2.1. Sample preparation

In this study, high-protein insect flour produced from *T. molitor* served as test material. Mealworm larvae were purchased from a local breeder (Futtertier-Shop.de, Eisenhüttenstadt, Germany), separated from frass by sieving, then packaged in freezer bags, subsequently inactivated by freezing and stored at −20 °C. *Tenebrio* flour was produced by pureeing frozen larvae with distilled water (1:1 *w*/w) at 4 °C, subsequent freezing at −20 °C, freeze drying (Christ Alpha 1-4, Christ Gefriertrocknungsanlagen, Osterode, Germany) and fine grinding in a coffee mill (Clatronic KSW 3307, Clatronic International GmbH, Kempen, Germany).

2.2. Cold atmospheric pressure plasma treatment

For semi-direct CAPP treatment of the insect flour, a surface dielectric-barrier air-discharge (SDBD) system was used. The setup is described in detail elsewhere (Bußler et al., 2015; Bußler, Steins, Ehlbeck & Schlüter, 2015). CAPP was generated by applying a sinusoidal voltage of 8.8 kV_{PP} at a frequency of 3.0 kHz using air as working gas. CAPP treatment of 4.75 g of *Tenebrio* flour was conducted in a Petri dish, which was fixed on a shaker at a distance of 12 mm below the plasma source. Thin layers of *Tenebrio* flour were evenly spread over the base area (50.3 cm^2) of the Petri dish in order to ensure homogeneity of treatments. Samples were agitated continuously (350 rpm) on the rotary shaker during exposure to CAPP for up to 15 min. The sample temperature during CAAP treatment was measured according to Bußler et al. (2015). Thermal load of the flour did not exceed 67 °C for the selected plasma application.

2.3. Thermal treatment

Thermal treatment of the *Tenebrio* flour was carried out in a drying cabinet by applying temperatures of 20, 40, 60, 80, 100, 120 and 140 °C. Glass Petri dishes (base area 50.3 cm^2) were preheated to the respective temperature subsequent to addition of the flour samples followed by thermal treatment of 15 min, which was terminated by removing the *Tenebrio* flour from the drying cabinet and transferring it into a cooled Petri dish.

2.4. Microbial analysis

Total viable count of the *Tenebrio* flour was analyzed by mixing and homogenizing 3 g of flour and 27 g of 0.1% casein–peptone-solution (CPS) in a sterile filter stomacher bag (Bag Mixer Interscience, St. Nome, France) at a speed level of 8 for 2 min. The homogenate was then serially diluted with CPS in Rotilabo®-microtest plates (96er U-profile, Roth, Germany), and 50 μL of each dilution was spread on plate count agar and incubated at 30 °C for 72 h to determine the number of colony forming units per g on a dry matter basis (cfu/g_{DM}). The detection limit of plate count analyses was 200 cfu/g_{DM}.

2.5. Mass loss and pH

Thermal and plasma-induced mass loss of the samples was determined by differential weighing. Shifts in pH were determined in the suspension of flour and 0.1% CPS (Inolab Terminal 740 pH measurement device, WTW, Weilheim, Germany). During further analysis, the pH values of the protein extracts in buffered systems (pH 4 and 10) were measured.

2.6. Characterization of techno-functional properties

2.6.1. Crude protein, crude fat and dry matter content

Crude protein content (N_{Kjel}, conversion factor 6.25) was analyzed using the method by Kjeldahl (Kjeldatherm Turbosog, Titrino plus 848, Gerhardt Analytical Systems, Königswinter, Germany), according to DIN EN 25663: Digestion and distillation (Kjeldahl Sampler System K-370/371) were conducted as described by the Association of German Agricultural Investigation and Research Institutions (VDLUFA, 1976). Crude fat content of the *Tenebrio* flour was determined according to the filter bag (Filterbags XT4, ANKOM Technology, New York, USA) method Am 5-04 (AOCS 2005). Dry matter (DM) content was determined via oven drying method (105 °C, 48 h).

2.6.2. Water (WBC) and oil binding capacity (OBC)

WBC of the *Tenebrio* flour was measured using the method by Smith and Circle (1978), modified by Quinn and Paton (1979). Therefore 0.5 g (±0.009 g) of *Tenebrio* flour was weighted into a centrifuge beaker and

stirred (60 s) with 2.5 mL of water using a propeller stirrer and an overhead agitator (Yellowline®, IKA® OST basic, New Jersey, USA). Following 20 min of centrifugation at 3900 *g* the supernatant was decanted and the beaker was put upside-down on filter paper for 60 min and was re-weighed. WBC was calculated following Eq. (1)

$$\mathrm{WBC}\left[\frac{g_{water}}{g_{DM}}\right] = \left(\frac{m_0 - m_1}{m_{0,DM}}\right) \quad (1)$$

where m_0 is the initial weight of the sample, m_1 is the final weight of the sample and $m_{0,DM}$ is the initial weight of the sample based on dry mass.

For the determination of the OBC, the method of Schwenke et al. (1981) was used. Therefore, 0.5 g (±0.009 g) of the *Tenebrio* flour was weighed into a centrifuge beaker and stirred with 2.5 mL of commercial rape seed oil two times for 60 s at 1000 rpm with a five-minute intermission in between (the stirring steps). Following steps were conducted as above mentioned. OBC was calculated similar to WBC.

2.7. Characterization of Tenebrio proteins and protein properties

2.7.1. Protein solubility

As *Tenebrio* proteins were found to show highest solubility at pH 10 and their pI was in the area of pH 4 (Bußler et al., 2016), extraction of the insect proteins was conducted using 0.1 M citrate buffer (pH 4) and 0.1 M borate buffer (pH 10) at a flour-to-solvent ratio of 1:25 under stirring on a rotary shaker (350 rpm). Protein extracts were centrifuged for 10 min (10,000 g, 4 °C) and the clear supernatants were subsequently analyzed.

The Biuret assay (Robinson & Hogden, 1940) was used for quantitative protein analysis.

Bovine serum albumin (Fluka, Buchs, Switzerland) was used as a standard (c_{Biuret} = 0–10 mg/mL in 2 mg/mL intervals). The assay consisted of 200 μL of the protein extracts reacting with 800 μL of Biuret reagent (45 min, 20 °C). The absorption maximum at 540 nm was measured against a blank value (solvent) by UV–vis spectrophotometer (BioPhotometer plus, Eppendorf, Hamburg, Germany). Yield of soluble insect protein was related to the respective dry matter of the *Tenebrio* flour.

2.7.2. Fluorescence measurement

Measurements of fluorescence emission spectra were performed using a PerkinElmer LS55 fluorescence spectrometer (Rodgau-Jügesheim, Germany) equipped with a pulsed xenon lamp and a red-sensitive photomultiplier (R928). The excitation wavelength was 280 nm and the fluorescence spectra were scanned in a wavelength range of 300–550 nm. A cut-off filter at 290 nm was placed in front of the emission monochromator (slit width 5). Differences in protein concentration and pH of the samples were equalized by dilution with 0.1 M phosphate buffer (pH 7). Measurement was conducted in triplicate using disposable semi-microcuvettes (Sarstedt Nümbrecht, Germany) against phosphate buffer as the blank.

2.7.3. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE according to Laemmli (1970) was used for determining the molecular weight distribution of the insect proteins. The samples (n = 3) were pooled and mixed in a ratio of 1:10 with sample buffer (0.0125 M Tris buffer at pH 6.8 containing 0.005 M EDTA at pH 6.8–7.0, 1% of sodium dodecyl sulphate, 10% of glycerol, 1% of 2-mercaptoethanol and 0.005% of Bromophenol Blue). Protein solutions were prepared by denaturing the proteins at 95 °C for 3 min prior to analysis. The gels were prepared using the vertical electrophoresis equipment (Mini-PROTEAN) from Bio-Rad (Bio-Rad Laboratories GmbH, Munich, Germany). PageRuler™. Unstained Broad Range Protein Ladder (Thermo Scientific, Lithuania) served as standard and 5 μL/10 μL of the samples were separated in 12% T gels. The band intensity was estimated following staining the gels with Coomassie Brilliant blue and corresponding de-staining protocols. Quantification was done using Quantity One 1-D Analysis Software, version 4.5.2 (Bio-Rad, Universal Hood II, Bio-Rad Laboratories, Segrate, Milan, Italy).

2.8. Statistical analysis

All treatments and analytical steps were conducted at least in triplicate. All data were statistically analyzed (ANOVA) with Statistica™ for Windows™ (version 9.0, Statsoft Inc., Tulsa, Okla.). Significant differences between means were determined by Turkey's HSD test ($p < 0.05$). In the figures, the mean variability of data was indicated by the standard deviation.

GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool), a freeware Add-in for Microsoft® Excel was used to fit the mean values of the inactivation data observed by thermal treatment of the *Tenebrio* flour (Geeraerd, Valdramidis, & Van Impe, 2005). The applied model describes the relation between survival and treatment time by

$$N = (N_0 - N_{res}) \cdot e^{(-k_{max} \cdot t)} + N_{res} \quad (3)$$

whereas N_0 is the initial microbial load [cfu/g_{DM}], N_{res} is the residual population density [cfu/g_{DM}], k_{max} is the specific inactivation rate [1/min] and t is the treatment time [min]. Besides the parameter values obtained, the standard errors of the parameter values, the Mean Sum of Squared Errors (SME) and its Root (RSME), the R^2 and the adjusted R^2 are automatically reported. The GInaFiT tool can help the end-user to communicate the performance of food preservation processes in terms of the number of log cycles of reduction rather than the classical D-value.

3. Results and discussion

3.1. Process induced mass loss, microbial inactivation and pH shift

Depending on the technology applied, specific effects on final mass, residual microbial load of the *Tenebrio* flour and on pH of protein extracts recovered thereof at pH 4 and 10 were detected (Table 1). Thermal treatment for 15 min at up to 140 °C induced linearly increasing mass losses of up to 0.11% whereby microorganisms were completely inactivated following treatment at 120 and 140 °C. The plasma-induced mass losses were found to be ten times higher. An almost linear (R^2 = 0.95) decrease in mass to 88.07% was detected. CAPP treatment reduced the initial microbial load of 7.72 $\log_{10}$ cfu/g_{DM} by 0.62, 1.00, 1.93, 2.53, 2.51, and 2.99 $\log_{10}$ cfu/g_{DM}, respectively. Plotting the heat induced inactivation kinetic enabled modelling of the data using a log-linear model with tailing covering shape which identified N_0 = 7.44 (±0.11) $\log_{10}$ cfu/g_{DM} and N_{res} 4.90 (±0.13) $\log_{10}$ cfu/g_{DM}. With R^2 = 0.9849 and a RSME = 0.1588, k_{max} = 0.75 (±0.08) was reported by the GInaFiT tool. In literature bi- and triphasic survival curves have been reported for various types of microorganisms including Gram-negative and Gram-positive bacteria, yeasts, and bacterial endospores on glass, agar, and polypropylene (Kelly-Wintenberg et al., 1999; Montie, Kelly-Wintenberg, & Roth, 2000). As stated by Montie, Kelly-Wintenberg, and Roth, the inactivation rate is dependent on the type of microorganism and the surface on which they were treated. In the case of multiphase inactivation using low-pressure plasma, the inactivation during the first phase can be mainly attributed to UV irradiation, whereas the slowed kinetic in the second phase is likely caused by erosion process by active species (Moisan et al., 2002). In accordance to these results, UV also plays a significant role in the case of CAPP treatment as applied in this study. However, besides lipid peroxidation by hydroxyl radicals, protein and DNA oxidation by oxygen radicals (Montie et al., 2000) are being suggested as the main inactivation mechanisms. Furthermore, the emitted reactive oxygen (ROS) and reactive nitrogen species (RNS) can cause irreversible oxidative damage to proteins, genetic

Table 1
Means (±sd) of thermal and plasma-induced mass loss and residual microbial load of *Tenebrio* flour as well as means of final pH of the casein peptone solution (CPS) and protein extracts prepared at pH 4 (1:25 *w/v* in 0.1 M citrate buffer) and pH 10 (1:25 *w/v* in 0.1 M borate buffer). Different letters indicate significant ($p < 0.05$) differences between means.

	Mass loss [%](n = 3)	Microbial load $\log_{10}$ N [cfu/g_{DM}](n = 6)	pH [−]		
			CP solution(n = 3)	pH 4(n = 9)	pH 10(n = 9)
T [°C]	Thermal treatment				
20	0.00^{a} (±0.00)	6.76^{a} (±0.07)	6.92^{a} (±0.03)	4.76^{a} (±0.05)	9.06^{a} (±0.08)
40	0.01^{b} (±0.00)	6.49^{a} (±0.25)	6.91^{a} (±0.02)	4.72^{a} (±0.01)	$8.94^{b,c}$ (±0.02)
60	0.02^{c} (±0.00)	5.30^{b} (±0.45)	6.93^{a} (±0.04)	$4.72^{a,b}$ (±0.01)	$8.97^{b,c,d}$ (±0.06)
80	0.05^{d} (±0.01)	5.27^{b} (±0.18)	$6.90^{a,b}$ (±0.02)	$4.70^{a,b}$ (±0.03)	8.98^{b} (±0.01)
100	0.06^{e} (±0.00)	4.19^{c} (±0.43)	6.89^{a} (±0.03)	$4.70^{a,b}$ (±0.03)	8.93^{c} (±0.01)
120	0.08^{f} (±0.01)	0.00^{d} (±0.00)	6.92^{a} (±0.01)	4.67^{b} (±0.02)	$8.95^{b,c}$ (±0.03)
140	0.11^{g} (±0.02)	0.00^{d} (±0.00)	6.91^{a} (±0.04)	4.64^{c} (±0.02)	$9.01^{a,d}$ (±0.01)
t [min]	Plasma treatment				
0	0.00^{a} (±0.00)	7.72^{a} (±0.14)	6.91^{a} (±0.03)	4.41^{a} (±0.00)	8.96^{a} (±0.07)
1	1.39^{b} (±0.09)	7.10^{b} (±0.13)	6.56^{b} (±0.04)	4.36^{b} (±0.02)	8.99^{a} (±0.05)
2.5	2.62^{c} (±0.09)	6.72^{c} (±0.11)	6.10^{c} (±0.06)	4.41^{c} (±0.00)	8.97^{a} (±0.13)
5	5.93^{d} (±0.47)	5.79^{d} (±0.31)	5.82^{d} (±0.05)	4.40^{d} (±0.00)	9.03^{b} (±0.02)
7.5	7.42^{e} (±0.24)	5.19^{e} (±0.32)	5.77^{d} (±0.04)	4.39^{e} (±0.01)	9.09^{c} (±0.02)
10	9.06^{f} (±0.44)	5.21^{e} (±0.20)	5.63^{e} (±0.02)	4.38^{f} (±0.01)	$9.07^{c,d}$ (±0.03)
15	11.03^{g} (±0.76)	4.73^{f} (±0.24)	5.32^{f} (±0.02)	4.38^{f} (±0.01)	9.06^{d} (±0.01)

material and fatty acids due to diffusion inside the microorganisms (Boudam et al., 2006; Laroussi & Leipold, 2004). However, the penetration depth of plasma-immanent species is limited. Pei et al. (2012) reported that a 25.5 μm thick biofilm of *Enterococcus faecalis* was inactivated by a hand-held plasma pen. In another study, Chen et al. (2014) also suggest a penetration depth of plasma of 10–50 μm into a biofilm.

Another point which needs to be taken into consideration is the shift in pH of the sample surface which is possibly induced upon CAPP processing of food or food components. In literature, the plasma-induced decrease in pH has been reported especially for liquid matrices (Segat, Misra, Cullen, & Innocente, 2015; Surowsky, Fischer, Schlueter, & Knorr, 2013). The results of this study clearly indicate that CAPP treatment also causes a pH reduction on surfaces of dry bulk materials as the pH of the unbuffered CP solution significantly decreased following shaking of the suspension from plasma-treated *Tenebrio* flour and CP solution. Neither in buffered systems at pH 4 and 10 nor following thermal treatment comparable effects could be detected. Similar effects on the pH of protein solutions following exposure of pea flour and pea protein to CAPP were reported by Bußler et al. (2015). In air plasmas the generation of ROS and RNS like NO and NO_x can be anticipated (Laroussi & Leipold, 2004). Acidification could be caused by the formation of nitrous acid (HNO_2) and nitric acid (HNO_3) from NO via NO_2, whereas acidification of solvents after addition of plasma-treated protein-rich solids may be attributed to the transition of non-covalently bound reactive plasma species or even split-off peptides or amino acids into the medium that either cause the acidic character or further may lead to the formation of acidic compounds. In case of CAPP treatment of whey protein isolate solution (Segat et al., 2015) a slight decrease in pH occurred after plasma treatment, which was statistically significant, particularly after 15 min. The authors noted that differences in acidity of plasma treated liquids may arise from several factors, including the volume of the treated liquid, the buffering capacity and the type of plasma source and inducer gas employed. However, the rather slight shift in pH detected in this study can be excluded as a possible cause for microbial inactivation induced by the CAPP treatment.

3.2. Impact on techno-functional properties of the Tenebrio flour

The initial crude protein and crude fat content, respectively, were neither affected by thermal nor by plasma treatment of the *Tenebrio* flour. Despite the relatively high mass loss following exposure to CAPP, the initial dry matter content only slightly increased whereas it was not affected by thermal treatments. In principle, a range of possible thermal and plasma-induced effects on each individual flour component and interactions between them are conceivable, which need to be taken into consideration as possible causes for the induced changes in flour functionality. Proteins represent the main ingredient of the *Tenebrio* flour used in this study and they mainly contribute to modifications in flour properties. As they substantially affect flavor and texture of foods, interactions of water and oil with proteins are very important in food systems. WBC of food proteins is dependent on intrinsic factors as amino acid composition, protein conformation and surface polarity/hydrophobicity, of which the two latter are impacted by food processing methods. Data obtained in this study show that thermal and plasma processing affected techno-functional properties of the *Tenebrio* flour (Fig. 1). Thermal treatment of the *Tenebrio* flour significantly reduced its WBC. In contrast, OBC was only marginally affected. An increase in OBC was detected following thermal treatment. CAPP treatment significantly decreased WBC of the *Tenebrio* flour, whereas its OBC was increased. However, no clear correlation between effect on OBC and CAPP treatment time could be detected.

Up to now, very little data is available on investigations of techno-functional properties of insect flours and their process-induced modification. Yi et al. (2013) analyzed foamability, foam stability and gelation of soluble proteins from five insect species. In former studies, water and fat binding capacities of different flour fractions from *T. molitor* and *Hermetia illucens* which changed upon processing of the insect larvae were reported (Bußler et al., 2016). Here, the techno-functional properties were dependent on insect species, composition (protein, fat and dry matter content) and process parameters applied. Impact on thermal and plasma treatment on the water and oil binding capacities are related to the process-induced effects on contained components or to interactions between them. During thermal treatment, *Tenebrio* proteins were likely denatured at high temperatures exposing more hydrophobic sites, which explains the decreasing WBC of the *Tenebrio* flour. The marginal effects on OBC are most likely caused by irreversible heat-induced denaturing which might have destroyed hydrophilic groups of *Tenebrio* proteins, thus increasing the proportion of hydrophobic residues. With regard to plasma-induced effects, numerous possible effects and potential reaction mechanisms have been reported in literature. It has been demonstrated that CAPP can cause changes in the protein structure. Reported results underline that CAPP can be successfully applied in order to selectively modify the structure of proteins and therefore, modify their functionality. Misra et al. (2015) explored the possible effects of CAPP as a means to change the structural and functional properties of strong and weak wheat flours and detected a voltage and treatment time dependent increase in the viscoelasticity of the dough produced

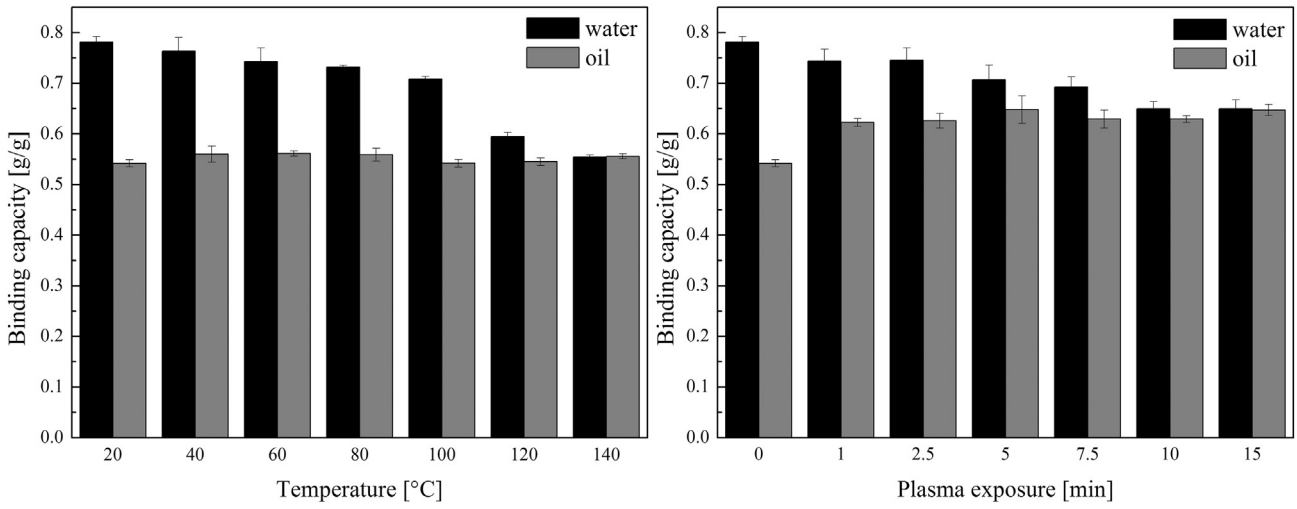

Fig. 1. Water and oil binding capacities of the *Tenebrio* flour following thermal (left, 15 min, for up to 140 °C) and CAPP (right, 3.0 kHz, 8.8 kV_{pp}, for up to 15 min) treatment. Values are calculated on a dry basis.

from the wheat flour. They attributed those effects to the alteration of the secondary structure of gluten proteins evidenced by FTIR spectroscopy analysis. Bußler et al. (2015) found that CAPP treatment modified protein- and techno-functional properties of different flour fractions from grain peas. Experiments using a pea protein isolate indicated that the reason for the increase in water and fat binding capacities in protein rich pea flour was based on plasma-induced modifications of the protein composition and structure as evidenced by changes in fluorescence emission spectra. Besides the reduction of free SH groups, mild oxidation in the proteins may be accompanied by an increase in carbonyl groups and surface hydrophobicity. These and other plasma-induced protein structure modifications may be responsible for the modification of protein properties. Nonetheless, the result of applying plasma on multi-component food systems is hard to predict and besides the plasma-induced effects on proteins, a wide range of interactions between different components needs to be taken into consideration.

3.3. Impact on protein solubility, structure and composition

Depending on treatment and process conditions applied, protein solubility, structure and composition were affected to a greater or less extent (Figs. 2-5). Among protein functionality properties, solubility is of primary importance due to its significant influence on the other protein properties. In general, proteins are required to have high solubility, in order to provide good emulsion, foam, gelation and whipping properties (Chan, Nakai, & Wood, 1985). The protein solubility is classified as a hydrophilic property as it relates to surface hydrophobic (protein–protein) and hydrophilic (protein–solvent) interaction and, in the case of food, the solvent is the water. When the secondary and tertiary structures of a protein are unfolded, the hydrophobic groups (initially inside the protein molecules) interact. Those hydrophobic interactions lead to aggregation, followed by coagulation and precipitation inducing a decrease in protein solubility compared to native protein. Quenching studies utilizing fluorescence of proteins can provide a wealth of information regarding the location of the intrinsic fluorophores within its macromolecular structure, thus providing structural information of the macro-molecule (Guo et al., 2014). Among the three fluorescent amino acid constituents of proteins, tryptophan (Trp) is the most abundant (Lakowicz, 2006). The contribution of phenylalanine (Phe) to the intrinsic fluorescence of protein is negligible by virtue of its low absorptivity in addition to a very low quantum yield. A variety of mechanisms lay at the basis of Trp fluorescence quenching by external or internal ligands. Although tyrosine (Tyr) has a quantum yield similar to Trp, the indole group of Trp is considered the dominant source of UV absorbance at ~280 nm

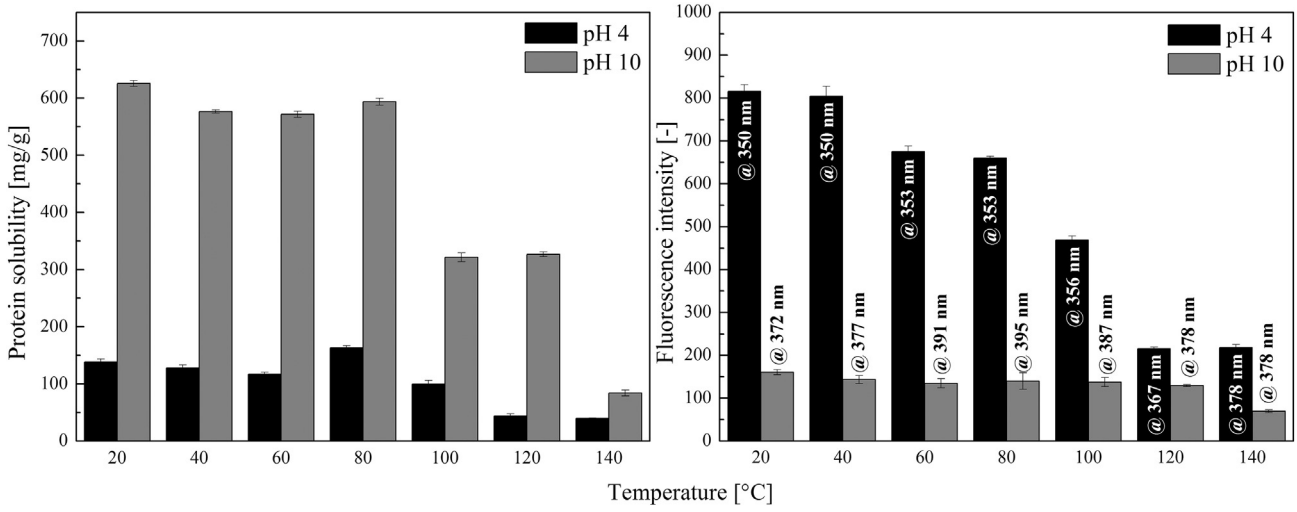

Fig. 2. Protein solubility (left) and fluorescence intensity (right) of proteins extractable from thermal treated *Tenebrio* flour at pH 4 and 10. Protein concentrations of the extracts were equalized prior to fluorescence measurement.

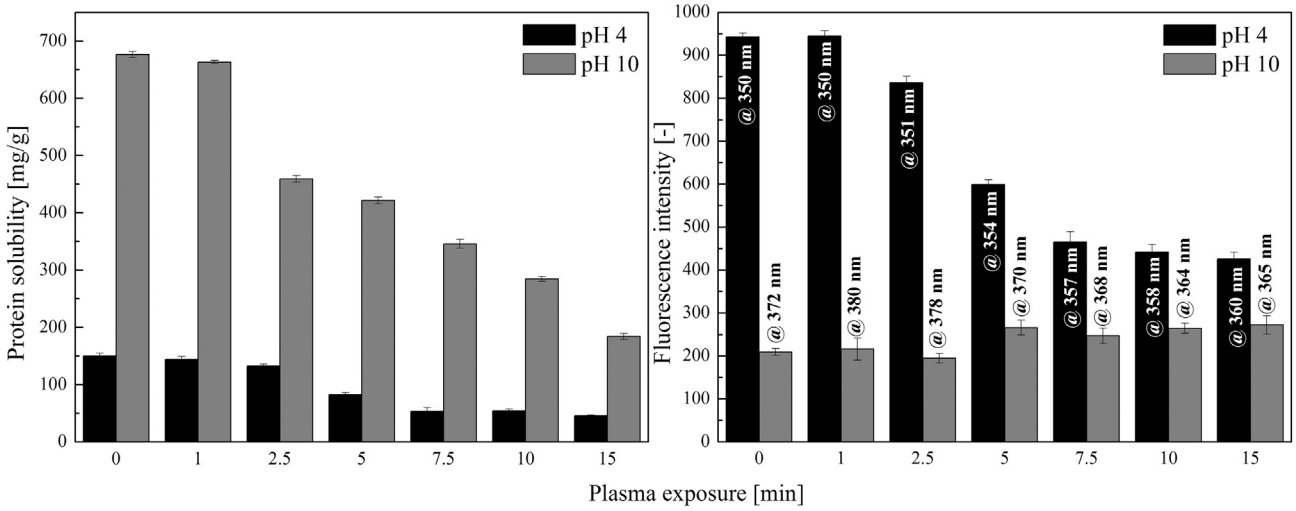

Fig. 3. Protein solubility (left) and fluorescence intensity (right) of proteins extractable from plasma treated *Tenebrio* flour at pH 4 and 10. Protein concentrations of the extracts were equalized prior to fluorescence measurement.

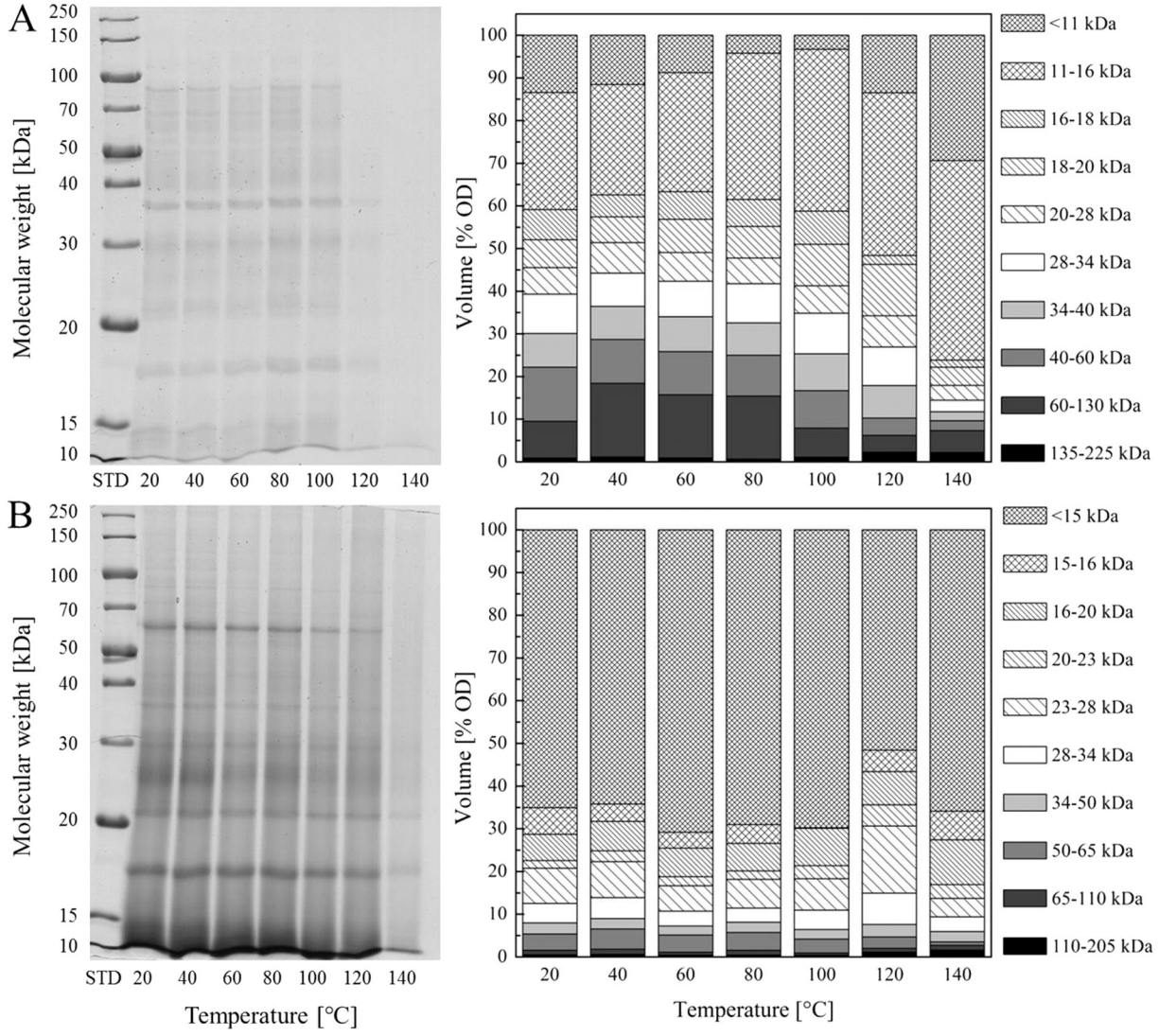

Fig. 4. Electrophoretic separation (left) and relative composition (OD = Optical Density) of soluble protein fractions (right) from *Tenebrio* flour depending on the treatment temperature following extraction at pH 4 (A) and 10 (B), $n = 3$.

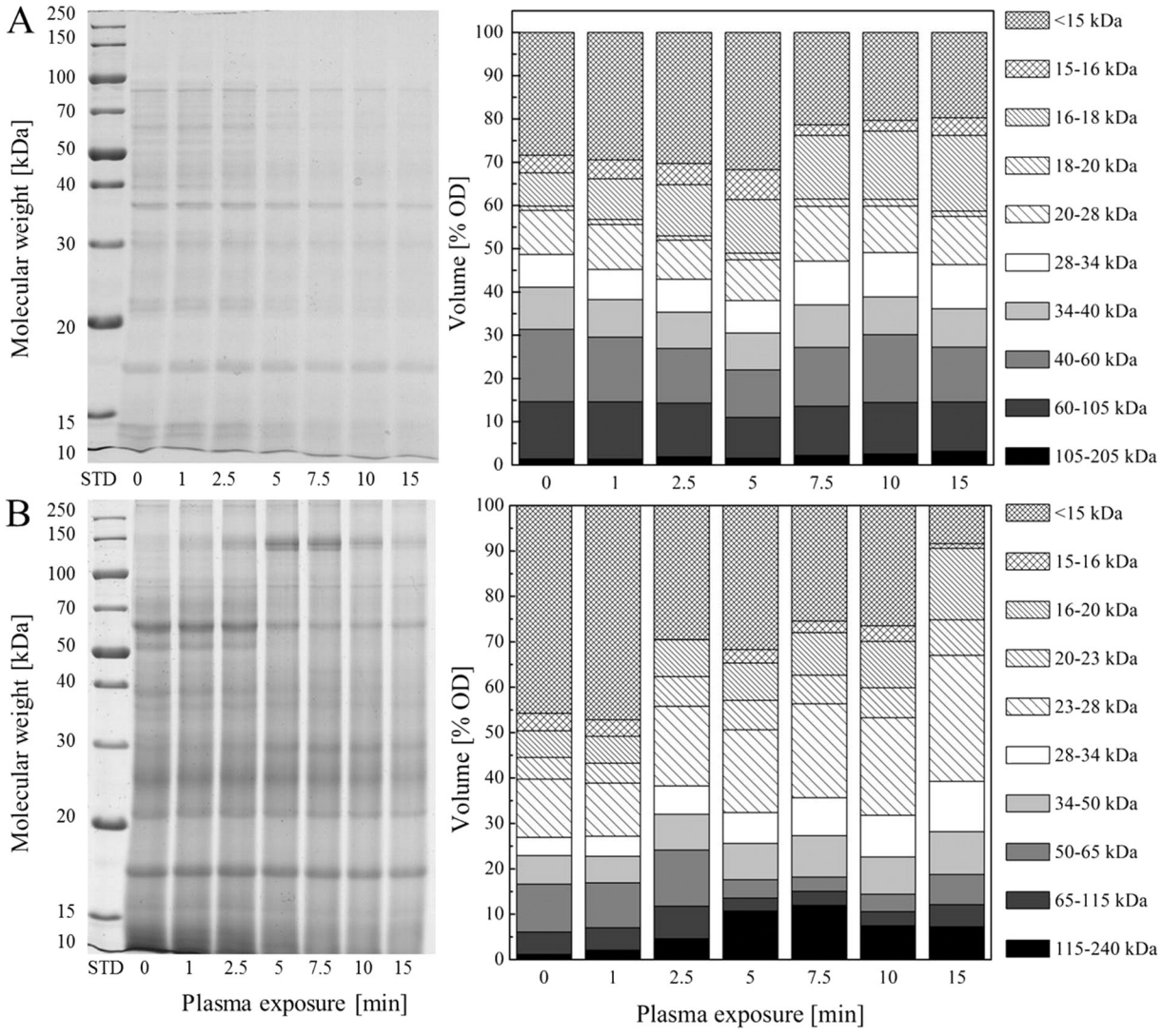

Fig. 5. Electrophoretic separation (left) and relative composition (OD = Optical Density) of soluble protein fractions (right) from *Tenebrio* flour depending on the CAPP exposure time following extraction at pH 4 (A) and 10 (B), $n = 3$.

and emission at ~350 nm in proteins (Teale & Weber, 1957). Analysis of process induced effects on the solubility of the proteins were supported by fluorescence analysis in this study in order to gain deeper knowledge on the different mechanisms of protein unfolding induced by thermal or CAPP treatment.

As expected, protein solubility was much higher at pH 10 where proteins were found to be highly soluble; pH 4 was identified as the isoelectric region of the *Tenebrio* proteins (Bußler et al., 2016). Exposure of the *Tenebrio* flour to elevated temperatures of up to 60 °C led to a decrease in protein solubility, whereas further increase in temperature to 80 °C increased the protein solubility (Fig. 2, left). Under both pH conditions elevating the temperature to 100 °C and above induced a sharp reduction in protein solubility at pH 10. Those effects were accompanied by changes in maximum fluorescence emission wavelengths (λ_{max}) and maximum fluorescence intensities (FI_{max}). Following protein extraction from heat treated *Tenebrio* flour at pH 4, an increase in temperature to 40 °C did neither significantly affect λ_{max} nor FI_{max} (Fig. 2, right). Increasing the temperature to 60 and 80 °C, respectively, led to a reduction of FI_{max} whereas λ_{max} shifted from 350 to 353 nm. A further reduction of FI_{max} and a marked shift of λ_{max} were detected upon increasing the temperature to up to 140 °C. In the case of protein extracts recovered at pH 10 FI_{max} was significantly lower for all tested temperatures and gradually decreased with increasing temperatures. Between 40 and 80 °C, λ_{max} shifted to bluer wavelengths and back to redder wavelengths with increasing temperatures to up to 140 °C. Temperature substantially influences the solubility of proteins. In general, it is increased at temperatures between 40 and 50 °C, when the temperature of the solution is elevated high enough for a given time, the protein is denatured. The temperature-induced effects are protein-specific. Using *Tenebrio* flour in all of the trials, the presence of non-protein impurities needs to be taken into consideration which may limit protein solubility as proteins may form complexes with for instance lipids that prevent their full solubilization. Hence one explanation for the increase in solubility of the insect proteins at elevated treatment temperatures of up to 80 °C may be attributed to weakened interactions between the proteins and other components as for instance fat. At higher temperatures, protein denaturation is caused by the effect of temperature on the noncovalent bonds involved in stabilization of secondary and tertiary structure (for e.g. hydrogen, hydrophobic and electrostatic bonds). The solubility decreased with increasing temperature and increasing protein denaturation due to thermal destruction of said bonds involved in the secondary and tertiary structure stabilization, as unfolding favors the interaction among the hydrophobic groups, reducing the protein–water interactions. In general, higher solubility is related with the presence of a low number of hydrophobic residues. Due to its aromatic character, Trp is often located in the hydrophobic core of protein

interiors, at the interface between two protein domains/subdomains, or at the subunit interface in oligomeric protein systems. In general, the more buried these residues are within the protein the less fluorescence quenching they will exhibit and the more exposed they will become to the solvent upon disruption of proteins' tertiary or quaternary structure as caused by treatments at high temperatures in this study. If a change in tertiary structure increases the exposure of tryptophanyl or tyrosinyl residues to the solvent, a decrease in fluorescence intensity is expected. Further, there will be less solvent induced fluorescence Stokes shift, which shifts the emission to redder wavelengths and over a broader spectral range. As total fluorescence of a protein is a mixture of the fluorescence from individual aromatic residues, several changes in the location and geometry of the individual fluorophore take place upon unfolding and restabilization of protein structures. On the other hand, there are also some proteins where tryptophan fluorescence gets quenched even though it is buried inside due to quenching by other residues. Results of this study clearly indicate that the changes in protein solubility due to thermal treatment of the *Tenebrio* flour were caused by unfolding and denaturation effects and further supports the hypothesis that the effects on techno-functional properties can be at least in part attributed to structural changes of the protein molecules contained in the flour.

CAPP treatment of the *Tenebrio* flour in general reduced the protein solubility (Fig. 3, left). Whereas CAPP treatment time of 1 min did not cause changes in λ_{max} and FI_{max}, prolonging the treatment time led to a decrease of FI_{max} (Fig. 3, right). This effect was accompanied by a shift in λ_{max} from 350 nm to 365 nm, thus also to redder wavelengths. Following extraction of the *Tenebrio* proteins at pH 10, a different behavior was detected as FI_{max}, which in general was lower compared to the values measured at pH 4, increased with increasing exposure to CAPP. Those effects were accompanied by an increase in λ_{max} from 372 to 380 and 378 nm following 1 and 2.5 min of CAPP treatment and a decrease to 365 nm with increasing exposure time to up to 15 min. Hence, a first shift of λ_{max} to bluer wavelengths induced by short time CAPP treatment was followed by a shift of λ_{max} to redder wavelengths with increased exposure to CAPP. The observed changes in protein solubility and fluorescence are most likely attributed to reactions between plasma-immanent species and functional protein groups at the surface of the *Tenebrio* flour particles. Plasma-induced protein oxidation can generate amino acid residue side chain modifications and changes in the protein polypeptide backbone, resulting in protein fragmentation, cross-linking, unfolding, and conformational changes (Segat et al., 2015; Shacter, 2000; Stadtman, 2006). ROS and RNS affect aromatic rings of amino acid residues of proteins. Furthermore, ROS, as atomic oxygen or OH radicals, may attack aromatic amino acids as Trp, which are sensitive to oxidation. Trp can be oxidized under formation of N-formylkynurenine and it has been suggested that it also might act as an antioxidant to a certain extent (Levine, Mosoni, Berlett, & Stadtman, 1996). This may be one possible explanation for changes in the fluorescence properties of Trp residues contained in the protein. Further it can be assumed that CAPP treatment induced an exposure of the hydrophobic amino acid residues normally present inside the protein structure. The red-shifted emission spectra at pH 10 following exposure of the *Tenebrio* flour to CAPP for 5 to 15 min may be attributed to unfolding or denaturation of protein structure inducing the exposure of the Trp to an aqueous environment as opposed to a hydrophobic protein interior. In some cases buried Trp residues are surrounded by aromatic amino acids, which results in so-called 'native' quenching of buried Trp. So in buried state, Trp displays emission maximum corresponding to buried Trp residues, but its quantum yield decreases. Consequently, in unfolded state, such proteins tend to exhibit good quantum yield and increase in overall fluorescence with characteristic red shift (Kosinski-Collins, Flaugh, & King, 2004). In contrast, a protein which contains a Trp which is exposed to the aqueous solvent will cause a blue-shifted emission spectrum if the Trp is embedded in a micelle (Caputo & London, 2003) or aggregate as one possible cause for the blue-shift of the emission spectra at pH 10 following exposure to CAPP. Different protein fluorescence maxima (and quantum yields) may further be caused by various ratios of two or more discrete classes of Trp residue contained in proteins which further have to be considered as a mixture of several protein components as also confirmed in this study (Figs. 4 and 5). Separation of the *Tenebrio* proteins via SDS-PAGE using 12% T gels resulted in wide ranges of protein bands. Ten major groups of *Tenebrio* protein bands could be distinguished. Depending on process and extraction conditions applied, the band patterns differed. As reported by Bußler et al. (2016) protein bands observed in the range between 14 and 32 kDa could possibly originate from cuticle proteins or chymotrypsin-like proteinase (24 kDa). The bands observed in the range between 32 and 95 kDa could stem from enzymes and other proteins, e.g. melanization-inhibiting protein (43 kDa), b-glycosidase (59 kDa), trypsin-like proteinases (59 kDa), and melanization-engaging types of protein (85 kDa). The bands with molecular weight > 95 kDa could possibly be linked to vitellogenin-like protein with a molecular weight of 160 kDa).

Band patterns significantly differed upon thermal treatment and variation of the solvent pH (Fig. 4A). At pH 4 thermal treatment at 40, 60 and 80 °C led to an increased proportion of fractions in the range from 60 to 130 kDa whereas at elevated temperatures of up to 140 °C it decreased whilst the amount of high-molecular fractions (135–205 kDa) increased. This was accompanied by increased proportions of low-molecular fractions (<11 kDa and 15 to 16 kDa). Following thermal treatment at 140 °C the protein extracts consisted of 72.3% low-molecular protein fractions compared to 40.7% following treatment at 20 °C. As previously described the solubility of the proteins was markedly decreased at temperatures above 80 °C which in the context of protein composition is most likely caused by the formation of insoluble aggregates separated from soluble low-molecular protein fractions by centrifugation. Those observations, which can be traced back to structural changes of the proteins, were supported by fluorescence emission spectra obtained in this study. In turn, alteration of the protein composition has to be considered as one reason for effects on protein fluorescence measured.

Protein extraction at pH 10 caused less pronounced effects on protein composition (Fig. 4B). Thermal treatment of the *Tenebrio* flour for up to 80 °C led to a slight increase in low-molecular fractions (<15 to 16 kDa). Further increase to 120 °C significantly increased the proportion of proteins ranging from 16 to 240 kDa whereas elevating the temperature to 140 °C led to a reduction of those fractions. In the case of treatment temperatures of 120 and 140 °C, the proportion of high-molecular protein fractions in the range between 115 and 240 kDa was slightly increased.

Comparing solubility at both pH values, by trend increasing the treatment temperature to 80 °C seemed to positively affect the protein solubility, which was considerably higher at pH 10. Extraction at pH 4 in turn led to higher stability of the protein composition upon thermal treatment at elevated temperatures of the *Tenebrio* flour. In general, thermal treatment increased the proportion of low-molecular protein fractions at pH 4 and increased the proportion of high-molecular fractions at pH 10.

The CAPP induced effects on protein composition significantly differed from those previously described (Fig. 5). An exposure to CAPP for up to 5 min led to an almost linear increase in proportion of protein fractions with molecular weights ranging from <15 kDa to 28 kDa (Fig. 5A) accompanied by a decrease in protein molecules in the range of 40 to 60 kDa for CAPP treatment of up to 2.5 min and protein extraction at pH 4. A 5 min treatment further induced a decrease from 13.6 to 9.9% in protein fractions in the range between 105 and 205 kDa which increased to 12.2% with increasing the treatment time to up to 15 min. Prolonging CAPP exposure to up to 15 min led to most pronounced changes in protein composition characterized by a decrease in low-molecular fractions ranging from <15 kDa to 20 kDa whereas the proportion of fractions ranging from 20 to 60 kDa was increased. Protein

extraction at pH 10 in turn caused contrary results demonstrated in a decreasing proportion of low-molecular protein fractions and an increase in high-molecular fractions with increasing treatment time (Fig. 5B). The maximum amount of protein fractions ranging from 115 to 240 kDa was reached following 7.5 min of treatment. Again, the plasma-induced impact on the protein structure and composition has to be considered as one reason for effects on protein fluorescence measured. The results confirm the specificity of plasma-induced protein modifications as solubility and fluorescence characteristics as well as composition of the *Tenebrio* proteins were not the same as induced by the application of elevated temperatures.

4. Conclusion

A dielectric barrier discharge induced CAPP was compared to thermal treatment during postharvest processing of *T. molitor* flour. A 3 log microbial reduction was achieved following exposure to CAPP for 15 min whereas equally long thermal treatments at 120 °C and 140 °C were found to completely inactivate the native microorganism flora. Both treatments affected the techno-functional properties of the *Tenebrio* flour and solubility, structure and composition of the proteins contained therein, but to a different extent. A *Tenebrio* protein extraction at pH 4 led to higher stabilities of the protein composition, towards thermal treatments at elevated temperatures in comparison to extraction at pH 10. In general the proportion of low-molecular protein fractions at pH 4 and the proportion of high-molecular fractions at pH 10 were increased, respectively. Prolonged exposure to CAPP for 15 min led to most pronounced changes in protein composition characterized by a decrease in low-molecular fractions ranging from <15 kDa to 20 kDa whereas the proportion of fractions ranging from 20 to 60 kDa increased. Protein extraction at pH 10 in turn caused contrary results demonstrated in a decreasing proportion of low-molecular protein fractions and an increase in high-molecular fractions. It appears that protein composition of the samples may have influenced the functional properties. The changes observed regarding protein solubility and fluorescence are most likely attributed to heat-induced protein unfolding and denaturation as well as to reactions between plasma-immanent species and functional protein groups at the surface of the *Tenebrio* flour and protein particles.

As the effects of CAPP application on multi-component foods are hard to predict, a wide range of interactions between different components needs to be taken into consideration. Findings regarding the plasma-induced impact on the characteristics of the *Tenebrio* flour and proteins therein indicate that the techno-functional properties can be effectively manipulated, but further research is needed to identify specific tools for tailoring them. Future studies and intense research will focus on the assessment of CAPP induced changes of single components and (the assessment) of plasma-induced interactions between two or multiple components. Especially with regard to the distribution of amino acid profiles and bioavailability of indispensable amino acids, further studies are required addressing bioavailability issues.

The results obtained in this study represent an important step towards the development of sustainable and microbiologically safe rearing, harvest and postharvest processing technologies. However, research efforts must be taken to evaluate the expenditure for the treatment for large quantities of food commodities at industry level also taking into consideration quality, safety, wholesomeness of food commodities to ensure high food and feed quality when exploiting insects as an alternative protein source.

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4.3 Complex food matrices: An initial approach towards process combinations

All the plasma-induced reactions, as amino acid oxidation, hydrogen bond disruption, and prosthetic group modification described for proteins, are also applicable on enzymes. Consequently, also the activity of enzymes can be influenced by CAPP treatment. As naturally occurring compounds in food, enzymes can be either desired or undesired. Since they catalyze reactions, which negatively affect food quality characteristics, most of them belong to the latter group. In this context, the enzymes polyphenol oxidase (PPO) and peroxidase (POD) are well known for being involved in enzymatic browning reactions including the loss in nutritional value. Lipases in turn are responsible for the formation of off-flavors through the decomposition of lipids. The majority of studies in food science literature dealing with the CAPP-induced inactivation of enzymes clearly demonstrated that plasma is capable of reducing enzyme activities, and some of them also tried to explain the underlying mechanisms. Meiqiang et al. [171] treated tomato seeds using a magnetized arc discharge plasma tube and were the first (and up to now, only) group who found an increase of POD activity induced by the exposure to CAPP. Activity of POD derived from maize roots was also subject of a study published by Henselova et al. [172], who found significant reductions in activity using a diffuse coplanar surface barrier discharge. Ke et al. [173] used an arc-discharge plasma with argon gas to investigate the impact on activity of horseradish peroxidase in PBS solution. POD was inactivated after a treatment time of 30 min. Several approaches were used in order to further identify the reactive species involved in POD inactivation. Using fluorescence measurement, it was found that the increasing peak at 450 nm after excitation at 330 nm is based on the destruction of heme, the cofactor responsible for POD activity. The authors concluded that H2O2 degraded heme into fluorescent products, while other factors, such as OH radicals, destroyed the structure of the enzyme. UV was found to be an additional factor, which accelerated the inactivation process in the presence of ROS. In a study conducted by Pankaj et al. [174], the kinetics of tomato POD inactivation by atmospheric air dielectric barrier discharge plasma inside a sealed package were studied. POD activity was found to decrease with both treatment time and voltage. In further studies, the impact of CAPP on dehydrogenase [171], malate synthase, isocitrate lyase, catalase and malate dehydrogenase [86], pectinolytic enzymes [175], lipase [176], catalase, dehydrogenase and superoxide dismutase [172], lysozyme [177], α-chymotrypsin [178] and lactate dehydrogenase [179] was investigated. While the majority of studies found decreased enzyme activities, Li et al. [176] have shown that lipase activity can increase during plasma exposure due to changes of the enzymes' secondary structure initiated by ROS. Investigations by Zhang et al. [179] give detailed information on the changes of the different secondary structure fractions in enzymes. According to their results, the α-helix content decreased upon plasma exposure, whereas the proportion of β-sheet regions, as well as random coils, increased. The finding of these studies support findings by Surowsky et al.

[180], who investigated CAPP impact on activities of PPO from mushroom, as well as POD from horseradish introduced into the upper layer of a (solid) model food system. They evidently showed that CAPP is capable of reducing the activity of both enzymes as a result of changes of their secondary structure fractions. In both cases, decreasing α-helix contents were accompanied by decreasing β-sheet contents. The results of fluorescence measurements supported the occurrence of structural changes. Decreasing fluorescence intensities of tryptophan, as well as a red shift, indicated a change of tryptophan surroundings to a more polar environment.

Most of the studies on plasma-induced enzyme inactivation were conducted in liquid or solid model systems containing the target enzymes. But food matrices are commonly composed of different amounts of macronutrients, as proteins, lipids, carbohydrates, and water representing, together with micronutrients, such as minerals and vitamins, essential components for the human diet. When observing the plasma-mediated reactions of these components isolated from others, and when the composition of reactive species applied is known, their behavior during plasma exposure might be comparably easy to predict. However, real food matrices are very complex multicomponent targets with different percentages of constituents, which influence the impact and induced process-interactions of the plasma applied. With its huge variety of reactive species the complexity of plasma-product interactions occurring becomes even more complex, when the gas discharge comes into contact with multicomponent systems, as complex food matrices. Compared to results derived from experiments using model food and single-component systems, a totally different behavior of the target compound could be caused in the presence of additional substances. Some of them act as scavengers and prevent other components from oxidation, while others act in the opposite way and enhance oxidation reactions [94, 125, 181]. Further, the formation of cross-links between different molecules, particularly proteins, can be mediated. Finally, the degradation and by-products formed often initiate further reactions, leading to multistep chain reactions. Taking into account that all of these reactions additionally depend on factors, such as pH, conductivity, state of matter, and macromolecular structure, underlines the challenge behind the application of plasma on food. Bringing plasma and complex food matrices into contact, the plasma-induced reactions occurring are dependent on the composition of both the plasma as well as the food matrix. Besides the reactions directly occurring between plasma-immanent species and molecules, reactions between subsequently formed radicals and molecules are induced and are summarized as molecule-radical interactions [182-184].

Especially reactions with antioxidants and water involved are of particular importance, since these compounds determine the progress of the reaction cascade initiated by ROS. Various molecules have scavenging ability against hydroxyl radicals, singlet oxygen, superoxide, and hydrogen peroxide. Minor components, such as ascorbic acid (vitamin C), tocopherol (vitamin E), flavonoids, and carotenoids are crucial antioxidative components contained in the food matrix protecting the matrix against ROS by different mechanisms. In general, sterically hindered phenolic groups contained in radical scavengers help to interrupt the radical transfer occurring during oxidation reactions by forming other, inert radicals finally stopping the reactions cascade and protecting other molecules from getting oxidized.

Vitamin E was identified as most efficient in scavenging peroxyl radicals in vivo and it was further reported that it is capable of scavenging hydroxyl radicals ($OH^{\cdot}$), singlet oxygen (1O2), and superoxide (O2). It particularly inhibits lipid peroxidation by breaking chain propagation both in vitro and in vivo [185].

Further, reducing agents, having very low redox potentials and thus, being oxidized much easier than other compounds, can act as antioxidants. As a consequence, ROS will preferably oxidize the reducing agents if present in the food matrix. One of the most widespread reducing agents in food is ascorbic acid. Exposing it to oxygen is generally followed by the Michaeles concept of a reversible two-step oxidation involving a free radical intermediate, resulting in the formation of dehydroascorbic acid [186, 187].

Besides these well-known antioxidants, several studies show that various carbohydrates are also capable of protecting other molecules from getting oxidized. Miller & Joslyn [188] and Kyzlink & Čurda [189] reported that fructose, glucose, and sucrose are capable of protecting ascorbic acid from getting oxidized by ambient oxygen. These results were confirmed for glucose, sorbitol, fructose, maltose as well as maltitol [190]. In addition, sugars were found to protect lipids from oxidation [191]. Compared to the antioxidant activity of individual phenolic antioxidants, it was decreased in mixtures prepared of sugars and phenolic antioxidants, such as tocopherol. The H-bonding activity of sugars/polyols hinders the H-donating activity of phenolic antioxidants.

Attri & Choi [178] investigated protective effects of ions on enzyme structure and activity against plasma-based degradation and found that chymotrypsin contained in buffer was protected by triethylammonium sulfate, as well as by triethylammonium dihydrogen phosphate (TEAP). Particularly the addition of TEAP resulted in retention of the enzymes' structure and activity.

These findings show that the result of applying plasma on multicomponent food is hard to predict, as basically every component can react with each other. Inactivation of enzymes and thereby preserving the nutritional benefits including available antioxidants is of special interest particularly in producing fresh-cut or dry produce from fruits and vegetables. Considering the fact that plasma-immanent species are able to react with every component in fruit and vegetables containing a complex mixture of ingredients quickly reveals that results obtained by using single-component model food systems are not simply transferrable to multi-component real food systems. The CAPP-induced inhibition of enzymes, as evidenced in studies using model systems, is perhaps not possible or enzyme activity may even be accelerated due to scavenging activity of antioxidants or the plasma-induced formation of educts being more susceptible to the conversion by intrinsic enzymes. Therefore, the efficacy of CAPP to inhibit enzymes needs to be investigated in detail for different fruits and vegetables further taking into account the plasma-induced secondary effects, which may be desired or undesired. In contrast to washing whole fruits or vegetables with PPW in order to inactivate undesirable or even pathogenic microorganisms on their peel, in case of producing fresh-cut fruits and vegetables, it generally makes sense to apply the CAPP treatment directly following cutting or as a final in-package treatment for stabilizing the fresh-cut produce

during storage. CAPP may also be applicable as a pre-treatment procedure during production of dried fruits and vegetables (Fig. 21).

Scientific work was conducted regarding the CAPP treatment of various foods including the treatment of fresh fruits and vegetables aiming at inactivating endogenous enzymes. Tappi et al. (2014) were the first ones, who investigated the impact of a low-frequency DBD with air as process gas on the activity of native PPO in fresh-cut apples. Some quality (soluble solid content, titrable acidy, color and texture) and metabolic parameters (PPO activity, respiration and heat production) were analyzed immediately following CAPP treatment and during storage. Besides a decreased tendency toward enzymatic browning, a linear decrease in PPO activity was evidenced with increasing treatment times. The authors conclude that the enzyme's loss in activity might be based on the action of OH and NO radicals on the amino acid structure. Whereas other qualitative parameters were only slightly affected, in general, CAPP treatment appeared to slow down the metabolic activity of the tissue.

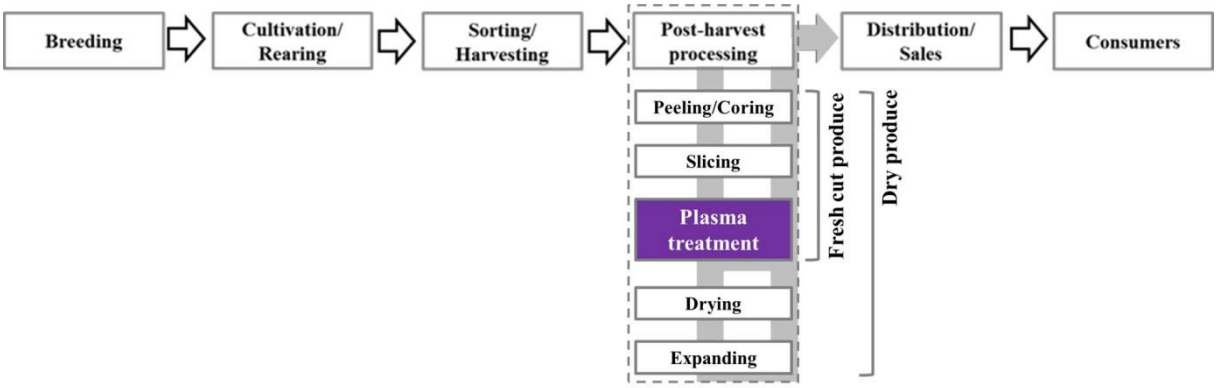

Figure 21: Potential integration of the CAPP technology into the value-added chain of fresh-cut and dry produce from fruits and vegetables.

Of course, the best way of maintaining the nutritional value of fruits and vegetables is keeping the product fresh, but most storage techniques require low temperatures, which are difficult to maintain throughout the entire distribution chain. Since the moisture content of fresh fruits and vegetables is higher than 80 %, they are classified as highly perishable commodities [192]. Dehydration offers a means of preserving foods in a stable and safe condition, as it reduces water activity and extends shelf-life. Further, fruits and vegetables are dried to enhance storage stability, minimize packaging requirement and reduce transport weight. Generally, the automatic production line for producing dried fruits and vegetables includes washing, peeling, (coring), slicing, drying, (expanding), and packing. Sliced fruits and vegetables are dried by drying machine, either by a hot air circulation drying oven or by a vacuum microwave drying machine. In most cases, drying process is carried out in conventional cabinet dryer (about 105 °C for about 120 min). As non-blanched fruits and vegetables are exposed to high temperatures in order to immediately inactivate the enzymes on the cut surface, color, flavor, and nutrient properties of the produced chips can be affected by caramelization or Maillard reactions. Drying at lowered temperatures may lead to accelerated enzyme activities, which can be prevented by blanching of the cut fruits and vegetables, which in turn is often accompanied with undesired losses of nutrients by leaching.

A pre-treatment with CAPP might offer an alternative approach to conventional blanching in order to reduce enzymatic browning reactions while preventing the loss of nutrients.

When considering CAPP treatment of real food matrices in an industrial scale, there are still large gaps in knowledge concerning the selection of proper plasma devices and parameters, as well as concerning the plasma-product-interactions and corresponding effects on quality attributes. To fill these gaps motivated parts of this work studying the impact of CAPP treatment on quality and stability fruits and vegetables as a possible pre-treatment to drying. For this purpose, each one representative of fruits (apple) and vegetables (potato tuber) each being used as dry products or ingredients was selected. Other relevant criteria for selecting apples and potatoes were the presence of PPO and POD as well as differences in composition and cell tissue structure. The context of raw material, plasma process parameters and quality attributes for post-harvest application are given in figure 22.

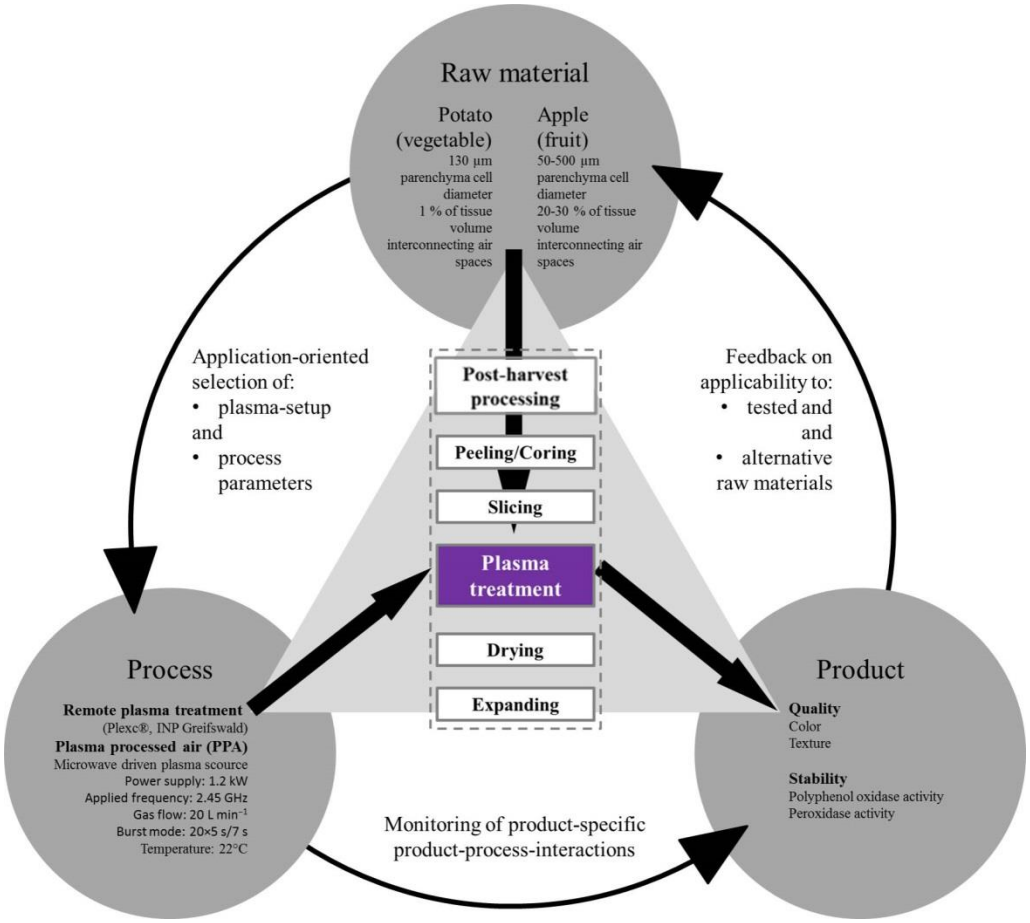

Figure 22: Schematic depiction of the relationship between raw material, plasma process parameters and product quality attributes for post-harvest CAPP applications.

The CAPP treatment of cut fruits and vegetables using a plasma jet system operating with noble gases is neither applicable for the homogenous treatment of the cell tissue surfaces nor with regard to the implementation of the treatment into industrial processing lines. Therefore, the use of a microwave driven plasma device enabling the remote treatment of bulky materials at ambient temperature and pressure conditions and cost-efficiently operating with air as the process gas was found to be the most promising plasma system.

In order to gain information on the plasma-induced product-process interactions, the initial concentration of plasma-immanent species, which in case of using air as process gas mainly consist of reactive NO_x species, as well as the way those species are degraded during exposure time were monitored. Further, the temperature in the treatment chamber, the resulting pH on the sample surfaces, as well as the product-specific visible penetration depth of the plasma, which each in turn contribute to the desired process goal of maintaining product quality by increasing its stability, were detected. Consequently, feedback on the applicability of the CAPP technology for

i. tested raw materials can be derived by evaluating the effectiveness of the treatment regarding the desired process goal and by
ii. identifying product-specific characteristics allowing transferability of the CAPP process.

With the long-term goal of combining plasma treatments with existing unit operations, as drying during processing of dried fruits and vegetables, it is mandatory to analyze the product- and process-specific effects on enzymatic stability, as well as quality parameters, such as color and texture.

This part of the thesis aims at providing insights into the inactivation of PPO and POD in fruits and vegetables by pre-drying CAPP treatment using a plasma device, which is promising for the application in industrial scale and thus, represents an initial approach towards process integration and combination (Section 4.3.1).

4.3.1 Pre-drying treatment of plant related tissues using plasma processed air: Impact on enzyme activity and quality attributes of cut apple and potato

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Pre-drying treatment of plant related tissues using plasma processed air: Impact on enzyme activity and quality attributes of cut apple and potato

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ABSTRACT

During post-harvest processing of fresh cut and dried fruits and vegetables, polyphenol oxidase (PPO) and peroxidase (POD) need to be inactivated or inhibited in order to avoid undesirable browning reactions and loss of sensorial or nutritional quality. To meet this goal, the application of plasma processed air (PPA) offers a promising "gentle" alternative to traditional methods, such as pasteurization or the addition of anti-browning compounds. Using ambient air as process gas instead of an expensive noble gas, such as argon, exhibits a substantial improvement for the development of large-scale plasmas at ambient pressure and allows the indirect treatment of larger goods within a remote exposure reactor. In this study the ability of PPA to inactivate PPO and POD in complex food matrices and its impact on quality parameters, such as color, texture and cell integrity directly after freshly cutting and during storage of warm air dried and freeze dried produce was evaluated.

The study evidently shows that PPA processing is capable of reducing the activity of PPO and POD in the freshly cut tissue from both apple and potato. Following exposure to PPA for 10 min the PPO activity was reduced by about 62% and 77% in fresh cut apple and potato tissue, respectively. POD, as the more temperature-stable enzyme, was even less stable upon PPA treatment for 10 min and was reduced by about 65% and 89% in fresh cut apple and potato tissue, respectively. Blackening of the potato tissue could be completely prevented by plasma treatment while a browning different from the habitual nature of enzymatic browning occurred upon exposure of the apple tissue to PPA. In both cases, the pH value on the tissue surface dropped to 1.5 while cell integrity and dry matter content were not significantly affected.

Industrial relevance: The quality and shelf life of freshly cut and dried fruits and vegetables greatly depend on the activity of naturally occurring enzymes which catalyze browning reactions at cut surfaces. This study shows that the application of PPA, as a promising nonthermal "pasteurization" technology, enables the inactivation of PPO and POD in complex food matrices. It further describes the impact of the PPA treatment on quality parameters of the freshly cut tissue from apple and potato and goes beyond on evaluating color, texture and enzyme activity in warm air dried and freeze dried tissue over a storage time of three weeks. The results contribute to the understanding and product-specificity of PPA-induced effects on quality and shelf life of fresh cut and dried fruit and vegetable produce and could be a basis for a possible industrial implementation.

1. Introduction

Modern day society is characterized by increasing health consciousness and the interest in the role of food for maintaining and improving human well-being and consumer health has grown over the past decades. Besides their nutritional and sensory properties, thus, foods are currently seen as active and protective agents and inter alia fresh-cut horticultural products stand out as convenient novel foods that cover most needs of a modern lifestyle as they combine technical content with an innovative food concept (Oliva & Barbosa-Canovas, 2005). The best way of maintaining their nutritional value is keeping the product fresh, but most storage techniques require low temperatures, which are difficult to maintain throughout the entire distribution chain. Since the moisture content of fresh fruits and vegetables is more than 80%, they are classified as highly perishable commodities (Orsat, Changrue, & Raghavan, 2006). Dehydration offers a means of preserving foods in a stable and safe condition as it reduces water activity and extends shelf-life. Further fruits and vegetables are dried to enhance storage stability, minimize packaging requirement and reduce transport weight. During the past two decades improving the quality retention of dried products by altering process conditions and/or pretreatments has been a major research goal (Cohen & Yang, 1995). The quality of dehydrated fruits and vegetables is dependent in part on changes occurring during processing and storage. Besides microbial spoilage,

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enzymatic browning is also a major concern on the extension of shelf-life of fresh-cut and dried fruit (Oms-Oliu et al., 2010) since residual enzyme activity in dried foods is an essential parameter affecting product quality and shelf-life. The activity of peroxidase (POD), whose primary function is to oxidize phenolic compounds by expensing H_2O_2, leads to negative flavor changes during storage and is further considered the most heat-stable vegetable enzyme and thus is also used as an indicator for successful blanching (Hendrickx, Ludikhuyze, Van den Broeck, & Weemaes, 1998). The enzymatic oxidation of phenols to quinones proceeds in the presence of oxygen, typically catalyzed by polyphenol oxidases (PPO). Quinones are then subjected to further reactions, leading to the formation of browning pigments (Jeon & Zhao, 2005; Nicolas, Richard-Forget, Goupy, Amiot, & Aubert, 1994; Ozoglu & Bayindirli, 2002) which was traditionally prevented by the use of sulfites. However, due to their potential hazards to health the use of sulfites on fresh-cut fruit and vegetables was banned in 1986 by the FDA (Buta, Moline, Spaulding, & Wang, 1999). In subsequent years, various alternative substances, such as honey, citric acid, ascorbic acid, calcium chloride, calcium lactate, calcium ascorbate and even fruit juices have been used to retard browning in fresh-cut fruit (Jeon & Zhao, 2005; Lozano-de-Gonzalez, Barrett, Wrolstad, & Durst, 1993; Oms-Oliu et al., 2010)—albeit with often limited success as it was difficult to achieve efficient browning inhibition.

Numerous studies dealt with innovative physical treatments (Ramos, Miller, Brandão, Teixeira, & Silva, 2013) such as high isostatic pressure (Schlüter, Foerster, Geyer, Knorr, & Herppich, 2009) being recently suggested for the application on some foods in order to inactivate enzymes without the degradation in flavor and nutrients associated with traditional thermal processing (Oliva & Barbosa-Canovas, 2005). Much attention has particularly been paid to pulsed electric fields (Barbosa-Cánovas, Góngora-Nieto, & Swanson, 1998; Knorr & Angersbach, 1998; Mertens & Knorr, 1992) and UV- or gamma-irradiation and packaging in modified atmosphere (Hassenberg, Huyskens-Keil, & Herppich, 2012; Lescano, Narvaiz, & Kairiyama, 1993; Poubol, Lichanporn, Puthmee, & Kanlayanarat, 2010; Sothornvit & Kiatchanapaibul, 2009) during recent years. However, the abovementioned methods could either not achieve the desired success or did affect the produce quality negatively or are even not completely harmless for consumers.

An innovative but largely unexplored nonthermal approach may be provided by the application of plasma processed air (PPA). Applying energy in the form of heat, voltage or electromagnetic fields to gas, ionization, excitation and dissociation reactions are induced which lead to the formation of various active components, such as radicals, UV light and charged particles, whereby reactive oxygen species (ROS), as atomic oxygen or OH radicals, and reactive nitrogen species (RNS) play a particularly important role (Laroussi & Leipold, 2004).

In food processing, the direct application of so-called “cold plasma”, as well as semi-direct or indirect treatment with thermal plasma is of interest as these can be used to treat the food at temperatures below 70 °C (Schlüter et al., 2013). Due to its nonthermal character and its operation under atmospheric pressure, cold plasma could be a suitable approach for the treatment of heat sensitive foods like fruits and vegetables.

Besides information about the denaturation of proteins by atmospheric pressure glow discharges (Deng, Shi, Chen, & Kong, 2007) the first available data concerning the impact of cold atmospheric pressure plasma on enzyme activity in a model food system were provided by Surowsky, Fischer, Schlüter, and Knorr (2013). They showed that cold plasma is capable of reducing the activity of the quality determining enzymes PPO and POD and suggested the possible inactivation mechanisms to be most likely based on a change in secondary structure of the enzymes. Since then, some publications appeared concerning the plasma-induced enzyme inactivation in fresh and fresh cut produce (Misra, Keener, Bourke, Mosnier and Cullen, 2014; Misra, Patil, et al., 2014; Tappi et al., 2014) using dielectric barrier discharge plasma setups. The plasma-induced impact of protein properties (Bußler, Steins, Ehlbeck and Schlüter, 2015) and flavonole glycoside profiles (Bußler, Herppich, et al., 2015) of peas was investigated further supporting the applicability of cold plasma for the treatment of fresh and dry agricultural produce.

The present study involves the use of a microwave-driven discharge to generate plasma processed air (PPA) as an indirect plasma application. The use of microwave-driven plasma torches is a well-established technique to generate plasma and has attracted the interest of a range of scientists in recent years because of its unique advantages (Baier, Herppich, Ehlbeck, Knorr, & Schlüter, 2015; Hertwig, Reineke, Ehlbeck, Knorr, & Schlüter, 2015; Schnabel, Niquet, Schlüter, Gniffke, & Ehlbeck, 2014). In this work, an evaluation of the potential use of PPA for the inactivation of PPO and POD in fresh cut and subsequently freeze and warm air dried produce from apple and potato tuber was conducted. The quality of the treated produce was evaluated based on change in color and textural properties, cell disintegration, surface pH and dry matter content.

2. Materials and methods

2.1. Preparation and storage of fresh cut and dried apple and potato tissue cubes

Apples of the Granny Smith variety and potatoes of the Milva variety (purchased at a local supermarket) were chopped into cubes (edge length 12 mm) immediately prior to exposure to PPA. Subsequent to the PPA treatment fresh cut samples were vacuum-packed in foil, frozen in liquid nitrogen and stored at −80 °C until further analysis. Warm air drying was carried out in a drying cabinet (65 °C, 24 h) whereas freeze drying was conducted at 0.5 mbar for 24 h (Alpha 1–4 LSC plus, Christ, Osterode, Germany). Storage of the warm air dried and freeze dried tissue cubes was carried out in sealed foil packages at 22 to 24 °C in the dark for 20 days.

2.2. Plasma processed air treatment

For the plasma ignition in air a microwave-driven plasma torch at a frequency of 2.45 GHz, a supplied power of approx. 1.2 kW and a gas flow of 20 L min −1 was used (PLexc®: Plasma excited, INP Greifswald). The device is consists of a microwave generator, a plasma source, a compressor, a humidifier, a device for oxidizing NO, a process chamber, a vacuum pump and of a control and regulating unit. The microwave generator supplies the plasma source with microwave energy generating hot plasma from the supplied air under atmospheric pressure (burst mode with an ignition/pause-cycle of 20 × 5 s / 7 s). The plasma gas emerging from the plasma source is cooled within a specified time to the point that a plasma-activated gas mixture with an NO_2 content of at least 0.5% is formed by means of the device for oxidizing NO. This plasma-activated gas mixture is humidified with water in a humidifier and further admitted into a process chamber containing the item to be treated (Krohmann et al., 2013). The process chamber was filled with the plasma processed air at room temperature (about 22 °C), resulting in nonthermal conditions within the treatment chamber. Further details regarding the plasma source set-up can be found elsewhere (Schnabel, Andrasch, Weltmann, & Ehlbeck, 2015). Apple and potato tissue cubes were put into baskets of perforated metal and placed into the exposure chamber. A spatial distance between the cubes was assured allowing a homogenous treatment of the overall sample surface. The cooled PPA was fed into the chamber, held for 2.5, 5, 7 or 10 min exposure time followed by venting with fresh air. The procedure was repeated three times for each exposure time in randomized order.

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2.3. Measurement of the quality parameters

2.3.1. Color measurement

The HunterLab-system was used to measure potential impact of plasma on the color of fresh cut and freeze dried apple and potato tissue surfaces during storage. A Minolta spectrophotometer (CM-2600D, Konica Minolta Inc., Osaka, Japan) was set at illuminant D65, 3 mm aperture, and 0° viewing angle. L-value (brightness), a-value (green–red axis), and b-value (blue–yellow axis) were taken for nine samples of each different plasma exposure times. According to Saricoban and Yilmaz (2010), the browning index (BI) was calculated as:

$$BI = \frac{[100 \cdot (x - 0.31)]}{0.17}$$

$$x = \frac{(a + 1.75 \cdot L)}{(5.645 \cdot L + a - 3.012 \cdot b)}$$

2.3.2. Texture measurement

The apparent modulus of elasticity (E) of the fresh cut apple and potato cubes was determined by means of a non-destructive quasi-static compression test (v = 10 mm min-1) using a universal texture analyzer (TA.XT.plus, Stable Micro Systems, Godalming, UK) equipped with a spherical steel body (d = 12.5 mm). The cubes were put on a flat horizontal base during measurement. Each cube was measured twice in the center of two different cube side surfaces. According to the formula given by ASAE (1999) E (MPa) was calculated from the deformation (D) at a maximum force (F) of 3 N as

$$E = \frac{0.531 \times F \times (1 - \mu)}{D^{1.5}} \times \left(\frac{4}{a} + \frac{4}{d}\right)^{0.5}$$

μ	Poisson-ratio = 0.49 (Mohsenin, 1986)
d	diameter of the steel body = 12.5 mm
a	edge length of the cubes = 12 mm

2.3.3. Impedance measurement

According to Angersbach, Heinz, and Knorr (1999), impedance measurement and the resulting calculation of the cell disintegration index were applied to characterize the degree of cell disruption induced by PPA treatment. The impedance analyzer SigmaCheck (Biotronix, Hennigsdorf, Germany) working in the frequency range of 10^3–10^7 Hz was controlled by SigmaCylinder software 2009 (Biotronix, Hennigsdorf, Germany). The measuring cell consisted of two cylindrical stainless steel electrodes (diameter 10 mm) which were separated to a distance of 10 mm by a polyethylene tube containing a cylinder of intact apple or potato tissue, respectively. A cell disintegration index between 0 (intact tissue) and 1 (complete cell rupture) was defined. In order to avoid any impact of variances in the respective tissue, every tissue cylinder was measured prior to and following exposure to PPA. The impact of PPA treatment was calculated as the difference in cell disintegration index. The standard errors represent the standard deviations of the results of at least nine independent measurements.

2.3.4. Surface pH and dry matter content

The pH values of the sample surfaces were measured by an Inolab Terminal 740 pH measurement device (WTW, Weilheim, Germany) equipped with a surface pH electrode. Dry mass (DM) was of the fresh cut and freeze dried tissue cubes was obtained after oven-drying at 105 °C for 48 h and their water content was calculated from fresh and dry mass.

2.4. Extraction process and enzyme activity assays

In order to extract the enzymes from the treated apple and potato tissue cubes, each two of untreated and treated, fresh cut or freeze dried cubes were inserted into cooled and sealable 50 mL reaction tubes. After adding 6 ml of 0.1 M PBS buffer (pH 6.5) samples were put in a refrigerator for reconstitution (1 h) followed by homogenization using a high-performance dispersion unit (Ultra turrax, IKA, Staufen, Germany) at 1350 rpm for two periods of 30 s each and an interval of 60 s on ice. Extracts were centrifuged at 4000*g* (4 °C) for 20 min and the clear supernatant was used for following analytical steps. The detection of PPO activity was based on the increase of absorbance at a wavelength of 420 nm, a temperature of 20 °C and a pH of 6.5 according to Siriphanich and Kader (1985). The measurement of the increase of absorbance (ΔE/s) was determined against a blank value by a Lambda 25 UV/Vis spectrophotometer (Perkin Elmer, Waltham, USA) for 2 min in equidistant fractions of time. The assay consisted of 250 μl of enzyme extract reacting in 1750 μl PBS buffer with 1000 μl of a catechol solution (Merck, Darmstadt, Germany, 0.1 M in 0.1 M PBS buffer, pH 6.5). The spectrophotometric detection of peroxidase activity was conducted following Stellmach (1988) by using pyrogallol which acts as a hydrogen donor and is oxidized to pyrogallin in the peroxidase catalyzed reduction of hydrogen peroxide. The increasing concentration of pyrogallin was measured by increasing absorbance (ΔE/s) against a blank value for 2 min in equidistant fractions of time. The assay consisted of 250 μl of enzyme solution reacting in 1950 μl dH_2O with 800 μl of a reaction solution (PBS buffer with 5 mM hydrogen peroxide and 0.1 M pryogallol). Enzyme activities are calculated as relative values which are obtained by dividing the measured activity following treatment by the initial activity

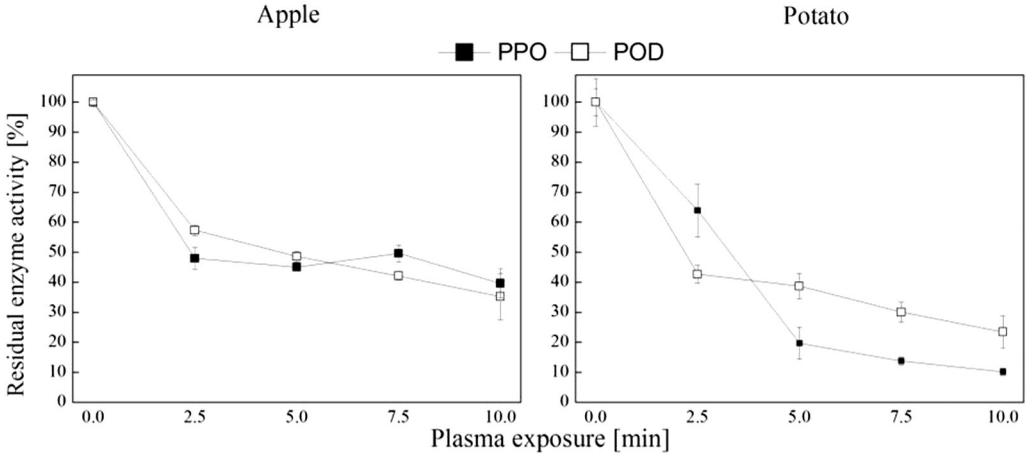

Fig. 1. Impact of PPA treatment on the residual enzyme activity of polyphenol oxidase and peroxidase in fresh cut tissue from apple and potato tuber.

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Table 1
Effect of PPA treatment on surface pH and dry matter content (DMC) of fresh cut und freeze dried tissue from apple and potato tuber. Different letters indicate significant ($p < 0.05$) differences between means.

PPA [min]	Fresh cut				Freeze dried		Warm air dried	
	pH		DMC [g/g]					
	Apple	Potato	Apple	Potato	Apple	Potato	Apple	Potato
0	$3.9^{a} \pm 0.0$	$5.9^{a} \pm 0.1$	$0.13^{a} \pm 0.03$	$0.20^{a} \pm 0.10$	$0.82^{a} \pm 0.11$	$0.88^{a} \pm 0.06$	$0.84^{a} \pm 0.03$	$0.95^{a} \pm 0.12$
2.5	$1.8^{b} \pm 0.2$	$1.9^{b} \pm 0.3$	$0.13^{a} \pm 0.01$	$0.18^{b} \pm 0.01$	$0.83^{a} \pm 0.09$	$0.88^{a} \pm 0.10$	$0.84^{a} \pm 0.08$	$0.93^{b} \pm 0.09$
5	$1.6^{b} \pm 0.3$	$1.6^{c} \pm 0.1$	$0.13^{a} \pm 0.01$	$0.18^{b} \pm 0.07$	$0.81^{a} \pm 0.03$	$0.89^{a} \pm 0.07$	$0.83^{ab} \pm 0.11$	$0.94^{b} \pm 0.09$
7.5	$1.5^{c} \pm 0.0$	$1.4^{d} \pm 0.1$	$0.13^{a} \pm 0.11$	$0.19^{ab} \pm 0.02$	$0.80^{b} \pm 0.09$	$0.89^{a} \pm 0.03$	$0.82^{bc} \pm 0.10$	$0.94^{b} \pm 0.10$
10	$1.5^{c} \pm 0.1$	$1.4^{d} \pm 0.1$	$0.12^{a} \pm 0.10$	$0.19^{ab} \pm 0.03$	$0.80^{b} \pm 0.03$	$0.89^{a} \pm 0.09$	$0.81^{c} \pm 0.07$	$0.93^{b} \pm 0.06$

of the untreated sample. The standard errors represent the standard deviations of the results of at least nine independent measurements.

2.5. Statistical analysis

All data were statistically analyzed (ANOVA) with Statistica™ for Windows™ (version 9.0, Statsoft Inc., Tulsa, Okla.). Significant differences between means were determined by Turkey's HSD test ($p < 0.05$). In the figures, the mean variability of data was indicated by the standard deviation.

3. Results

3.1. Fresh cut apple fruit and potato tuber tissue

Exposure of freshly cut apple and potato tuber flesh to PPA for up to 10 min resulted in a decrease of PPO and POD activities (Fig. 1). The inactivation kinetics of PPO and POD thereby were shown to be biphasic, as they were characterized by a steep decrease in residual enzyme activity after exposure to PPA for 2.5 and 5 min, respectively, followed by an abrupt flattening of the inactivation progression with increasing the exposure time to 7.5 and 10 min. Nonetheless, product and enzyme specific differences were observed. In apple flesh, PPO activity was reduced to 48% while it remained almost 16 percentage points higher in potato tuber flesh after exposure to PPA for 2.5 min. While for apple flesh increasing the treatment time to 10 min only resulted in a slightly improved PPO inactivation to 42%, applying identical process conditions to the flesh from potato tubers led to the inhibition of PPO activity to 10%. Whereas a first rapid decrease in PPO activity to 20% was achieved after exposure of potato cubes to PPA for 5 min, the residual activity slowly approached values of around 10% in the second stage. In comparison, POD activity in potato tuber flesh was less affected resulting in a reduction to 39, 30 and 24% following exposure to PPA for 5, 7.5 and 10 min, respectively. Compared to PPO, the first stage, representing a rapid loss of enzyme activity, was slightly shorter (2.5 min), whereas the second stage did indicate a further decrease of POD activity by 19%. POD inactivation kinetic in apple flesh was quite similar to that of PPO. Exposure of the fresh cut cell tissue from apples and potatoes to PPA led to a decrease in surface pH which was shown to be greatly dependent on the treatment time (Table 1). Starting from pH 3.9 and 5.9 on the surface of apple and potato, respectively, in both cases a sharp decrease in surface pH was triggered by exposure to PPA for 2.5 min followed by a rather slight decrease to 1.5 and 1.4, respectively, for apple and potato following plasma treatment for 10 min.

Despite the use of dry air (below 32% relative humidity) as working gas, no impact of the plasma treatment on the dry matter content (Table 1) of the fresh cut apple and potato tuber cubes was detected. However, exposure to PPA partially influenced the texture of the cell tissue. Whereas the modulus of elasticity of apple cubes was not significantly affected by exposure to PPA (Fig. 2), it was significantly reduced from 1.56 (0 min) to 1.36 (5 and 7.5 min) and 1.3 MPa (10 min) in case of potato tissue.

Depending on the degree of process intensity, cell disintegration index was only slightly increased to a maximum of 0.12 (Fig. 3) within potato tuber tissue which is negligible and, consequently, cannot have caused textural changes obtained. Cell disintegration index of apple tissue was increased to a maximum of 0.16 after exposure to PPA for 5 min and did not change by increasing the treatment time to up to 10 min.

Also with regard to color changes, product specific effects were apparent upon PPA treatment (Fig. 2). While exposure to PPA did not affect the browning index of freshly cut potato tuber cubes (about 45 for treated and untreated samples), it was increased from 30 to 78 (2.5 min) and 75 (5, 7.5 and 10 min) for freshly cut apple cubes, whereas no significant differences were observed with varying exposure times.

3.2. Freeze dried and warm air dried apple fruit tissue

Enzyme activities of PPO and POD were reduced upon exposure to PPA in freeze dried and warm air dried apple flesh cubes over a storage time of 19 days (Fig. 4). During the first 5 days of storage enzyme activities in untreated freeze dried apple cubes slightly decreased to 92%

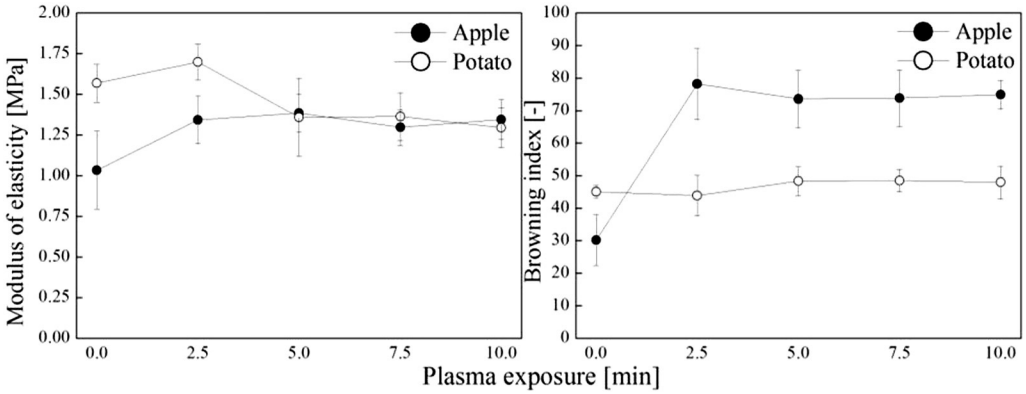

Fig. 2. Impact of PPA treatment on modulus of elasticity and browning index of fresh cut tissue from apple and potato tuber.

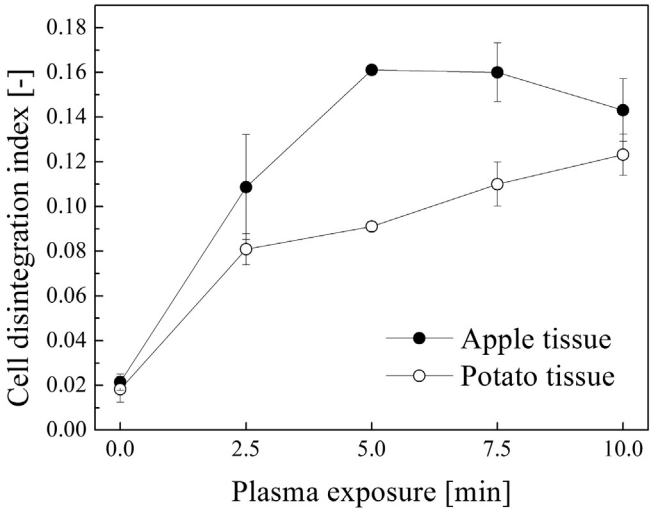

Fig. 3. Impact of PPA treatment on cell disintegration index of fresh cut tissue from apple and potato tuber.

followed by a reduction to 38 (PPO) and 45% (POD) until day 12. PPO activity decreased to 18%, whereas POD activity decreased to 14% during the whole storage period of 19 days. In contrast, PPO and POD activities remained almost continuously around or below 10% following all plasma treatment times over the entire storage period.

In warm air dried control apple flesh a different inactivation behavior of PPO and POD was observed. Enzyme activities in untreated samples remained nearly constant at about 100% over the storage time of 19 days. Exposure to PPA reduced the activity of both enzymes. Starting from 60% at day 1, PPO activity further decreased to 52% within 5 days of storage and remained stable until day 19 following exposure to PPA for 1 min. With increasing treatment time, PPO activity was further decreased, but less effective compared to freeze dried apple cubes. Dependent on the exposure time to PPA, PPO activity was significantly reduced during storage.

Except for a 10 min exposure to PPA, dry matter contents of the apple tissue cubes were found to be slightly higher in comparison with the values obtained for the freeze dried samples (Table 1). Compared to the untreated samples, dry matter contents of the apple tissue cubes were slightly increased by 2.9% following exposure to PPA for 7.5 and 10 min and subsequent freeze drying. Residual moisture content did not change upon storage (data not shown).

Well known and described differences in color and textural properties upon freeze drying and warm air drying were also apparent in this study. Regarding the textural properties of the untreated freeze dried and warm air dried samples significant differences were determined (Fig. 6). Modulus of elasticity was 0.3 MPa following freeze drying and did not significantly change over the entire storage period whereas for warm air dried samples the initially detected value of 1.4 MPa decreased to 0.55 MPa within 5 days of storage and remained constant until day 19. PPA treatment significantly affected the texture of freeze dried and warm air dried apple cubes. Effects were less pronounced for warm air dried apples but indicated a softening of the cubes compared to the untreated samples which became apparent by an increase of modulus of elasticity to 1.0 MPa (10 min) at day 1. E sharply dropped to 0.5 MPa (0.6 MPa) for plasma treated (untreated) warm air dried apple cubes after 5 days of storage followed by a further decrease to 0.3 MPa for samples exposed to PPA for 7.5 and 10 min. In case of PPA treated samples E was continuously lower (0.1 MPa) compared to untreated samples (0.3 MPa) also showing in noticeable softness and stickiness.

As expected, freeze dried apple cubes appeared lighter compared to warm air dried samples directly after drying. This also became apparent in the values calculated for the browning index (Fig. 6). Whereas it was 18 for untreated freeze dried apple flesh cubes, it amounted to 34 for warm air dried samples. This effect was not apparent in freeze dried apple cubes, as the browning index did not significantly change over the entire storage time. Further, no significant impact on browning index was detected for plasma treated apple cubes during storage but as already apparent for fresh cut samples, browning index was overall increased to 50 to 60 following freeze drying and to 55 to 65 following warm air drying of plasma treated samples.

3.3. Freeze dried and warm air dried potato tuber tissue

Enzyme activities in freeze dried and warm air dried potato tuber cubes were reduced upon storage for 19 days (Fig. 5) but inactivation showed to be enzyme-specific and dependent on applied drying technology. Both, PPO and POD activities decreased over the entire storage period leading to residual enzyme activities of 15 (PPO) and 25% (POD), respectively. PPA treatment led to a dose-dependent PPO and POD inactivation which was in case of PPO far less pronounced compared to the inactivation obtained in apple flesh. Also in case of plasma treated samples POD activity decreased over storage duration whereas most effective inactivation was achieved by exposure to PPA for 10 min. At the end of storage PPO activity was at a comparable level for all PPA treatment times. In contrast, POD activity was reduced dose-dependently. On all subsequent days of storage residual POD activity was at a level of about 2% for all treatment times.

Similar to the effects obtained for apple flesh cubes, PPO and POD activities were reduced less effectively compared to those in freeze dried potato cubes. In comparison, at day 1, PPO activity in warm air dried potato tuber cubes was substantially reduced and further decreased over 19 days of storage. Exposure to PPA led to a more effective reduction of POD activity subsequent to warm air drying. At the end of the storage period residual POD activities were further reduced.

Compared to the untreated samples, exposure to PPA did influence the dry matter content neither in freeze dried nor in warm air dried cubes of potato tuber (Table 1). Contrary to the results obtained for apple flesh cubes, dry matter contents in warm air dried potato cubes were significantly higher (0.95 g/g) than those of freeze dried samples (0.88 g/g).

Textural properties of the freeze dried and warm air dried samples changed upon storage (Fig. 7). Regarding the untreated samples, E was 0.6 MPa following freeze drying and did not significantly change during 5 days of storage whereas for warm air dried samples the initially detected value of 2.0 MPa dropped to 0.9 MPa. Further storage led to a decrease in E to 0.3 MPa in untreated freeze dried potato tubes while it remained steady for the warm air dried samples. In both cases E was lower for the plasma treated cubes over the entire storage period.

Similar to the freeze dried apple cubes the freeze dried potato cubes appeared lighter compared to warm air dried samples directly after drying. This resulted in browning indices of 12 (freeze dried) and 23 (warm air dried), respectively, which remained constant over the entire storage period in both cases (Fig. 7). In comparison, for samples exposed to PPA lower browning indices were detected resulting in values of 5 (freeze dried) and 15 to 18 (warm air dried). Here again, no alteration in color occurred upon storage.

4. Discussion

The observed biphasic behavior of enzyme inactivation might be based on the enzyme specific effect of PPA, as PPO and POD must be considered as mixtures of several enzymes, but also on the presumably very low penetration depth of plasmas (Xiong, Du, Lu, Cao, & Pan, 2011). Consequently, the first, rapid stage of enzyme inactivation might be attributed to a good accessibility of the enzyme to the plasma, and the slower second phase similarly to worsened accessibility, as presumed by Surowsky et al. (2013). Their results, which depicted a strong correlation between the losses of enzyme activity and the losses of α-helical structure, supported that the observed change in secondary structure is

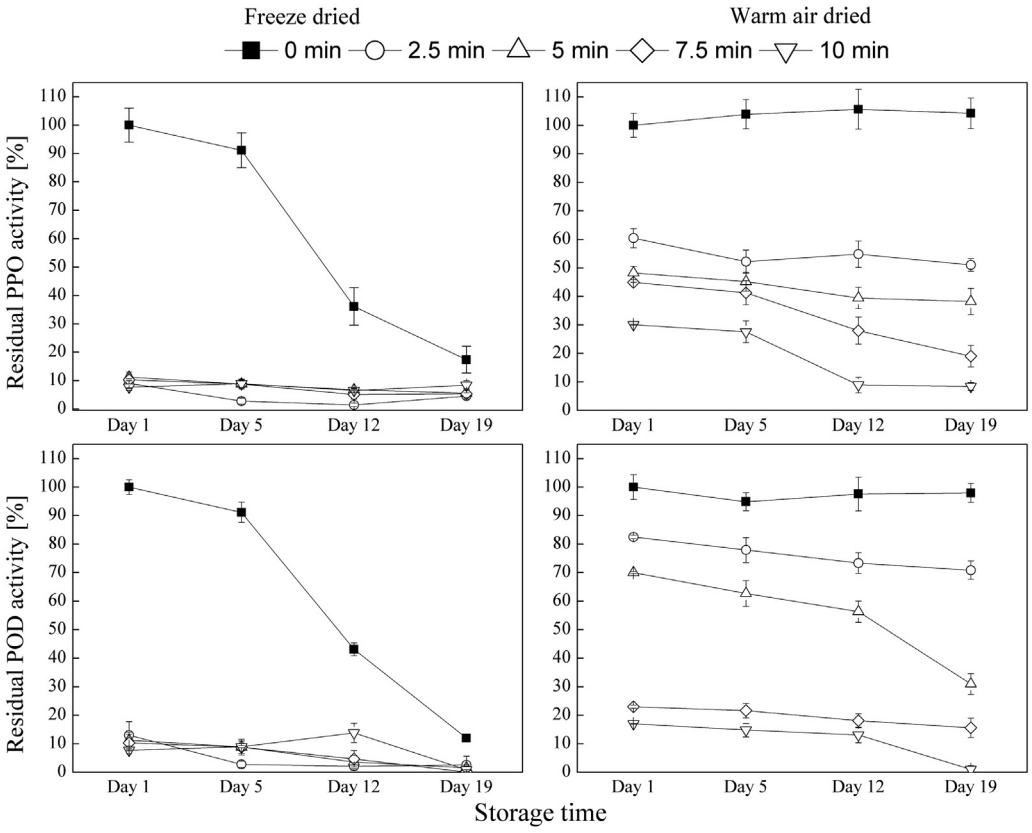

Fig. 4. Impact of PPA treatment on the residual enzyme activity of polyphenol oxidase and peroxidase in freeze dried and warm air dried tissue from apple over a storage time of 19 d.

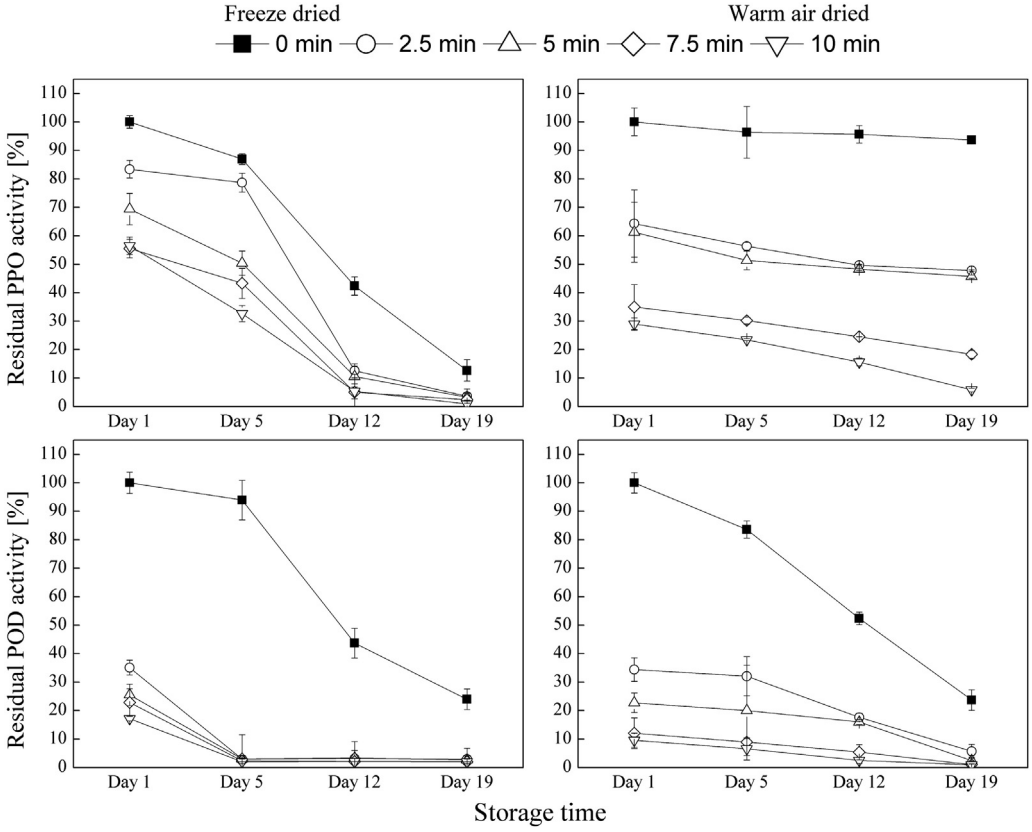

Fig. 5. Impact of PPA treatment on the residual enzyme activity of polyphenol oxidase and peroxidase in freeze dried and warm air dried tissue from potato tuber over a storage time of 19 d.

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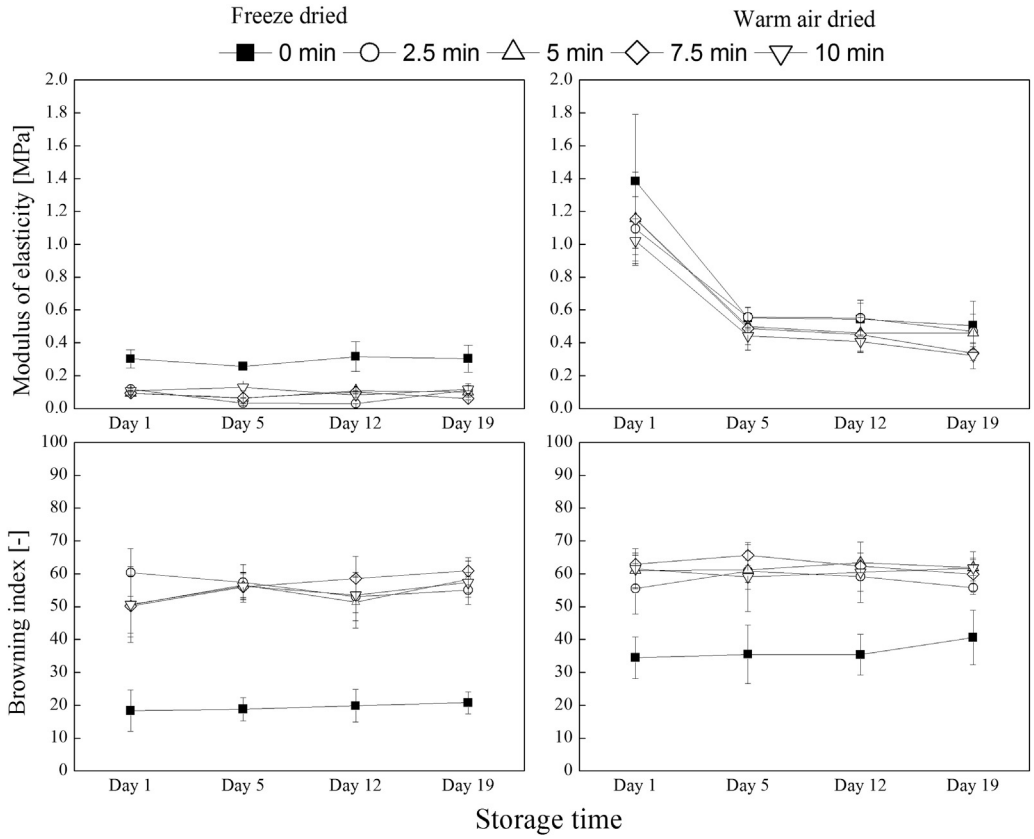

Fig. 6. Impact of PPA treatment on modulus of elasticity and browning index of freeze dried and warm air dried tissue from apple over a storage time of 19 d.

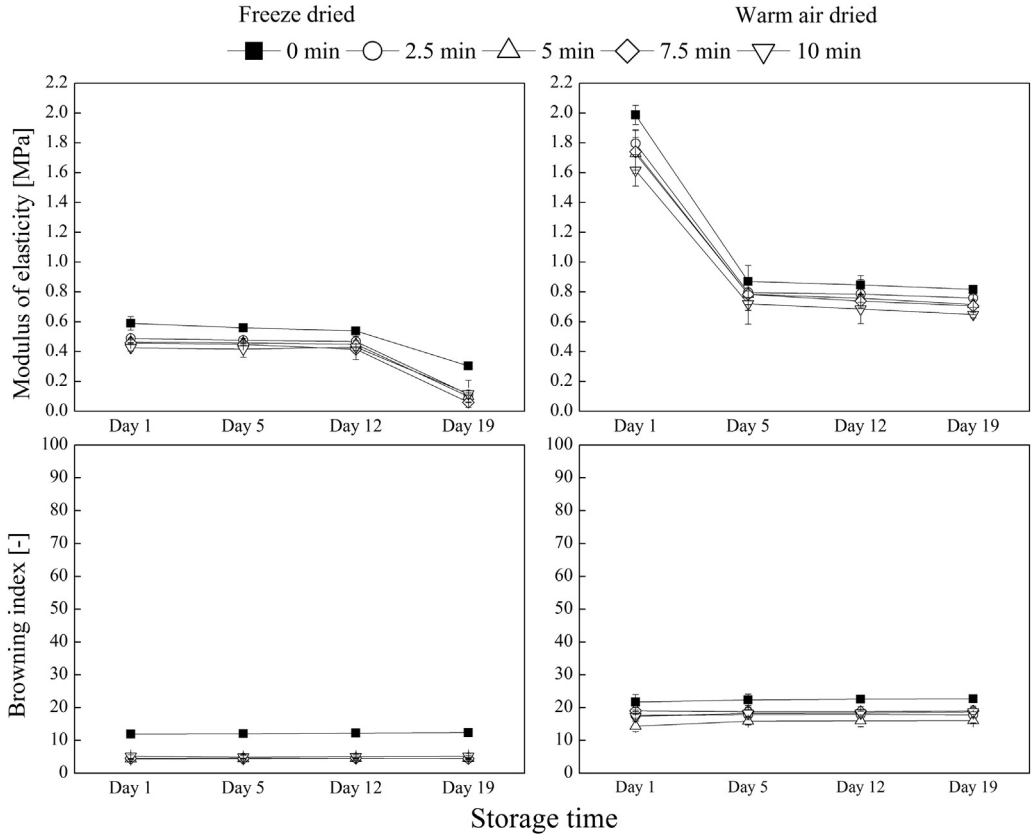

Fig. 7. Impact of PPA treatment on modulus of elasticity and browning index of freeze dried and warm air dried tissue from potato tuber over a storage time of 19 d.

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the main reason for the loss of enzyme activity. In comparable studies, Tappi et al. (2014) measured a significant and roughly linear decrease in PPO activities to 88, 68 and 42%, by exposure of fresh cut apples to plasma for 5 + 5, 10 + 10 and 15 + 15 min using a dielectric barrier discharge plasma device and air as the working gas. A mechanism of reaction between plasma generated reactive species and proteins was suggested by Takai, Kitano, Kuwabara, and Shiraki (2012) in order to explain the inhibitory effect of cold plasma on tomato peroxidase. They primarily attributed conformational changes in particular to the complex plasma chemistry initiated by plasma-inherent ROS and UV photons and hypothesized that OH, O_2^-, HOO and NO radicals induced chemical modifications of chemically reactive side-chain of the amino acids, such as cysteine, aromatic rings of phenylalanine, tyrosine, and tryptophan, that consequently lead to a loss of enzyme activity. Hayashi, Kawaguchi, and Liu (2009) described a similar mechanism for decomposition of C—H, C—N and N—H bonds of proteins.

In this study, UV photons only play a subordinate role as chemical reactions mainly based on ROS and RNS are expected by using a microwave driven plasma setup and dry compressed air as the working gas. Schnabel et al. (2014) analyzed the composition of microwave PPA using identical experimental conditions via mass spectrometry and showed that 2.7% of the working gas is converted into NO_2, NO, and a mixture of HNO_2, HNO_3, CO_2, and H_2O. As nitric oxide (NO) cannot coexist with ozone or atomic oxygen the formation of O_2, NO_2 and NO_3 proceeds via oxidation reactions (Surowsky, Schlüter, & Knorr, 2014). In the context of gas–liquid interfaces reactive nitrogen species (RNS) are also of interest as for instance through the reaction of NO with OH radicals, nitrite (NO_2^-) and nitrate (NO_3^-) are formed, which might influence the pH of the liquid or, as in this study, the surface pH of moist foodstuffs. In accordance to the enzyme classification by Miyagawa, Sannoe, and Suzuki (1964), and as already shown by Surowsky et al. (2013) the results obtained in this study confirm the incomplete inactivation of PPO and POD in fresh cut apple and potato tuber flesh. Based on this, the reduction of PPO and POD activities, which seem to be specific to the product and the drying technology chosen, can be described as irreversible. As all apple and potato flesh cubes exposed to PPA and subjected to either freeze drying or warm air drying were taken from the same batch, differences obtained concerning the residual enzyme activities are most likely due to the combination of plasma treatment and the drying technology chosen. Different process conditions applied and consequential structural changes in the secondary structure of the enzymes during freeze drying and warm air drying may be the reason for the reversible or irreversible inactivation of enzymes (Adams, 1991; Luyben, Liou, & Bruin, 1982).

The marginal effects on dry matter contents of apple and potato tuber tissue demonstrate that the plasma-induced increase in cell disintegration index did neither enhance the removal of water from apple during drying processes applied, nor led to higher water retention. However, those results do not allow drawing conclusions on the effects on the drying courses which may be influenced by the plasma pretreatment.

As the dry matter content of the apple and potato tuber tissue was not influenced by exposure to PPA, the detected effect on the modulus of elasticity was not caused by water evaporation effects but may probably be attributed to the differences in cell tissue nature of apple and potato tuber flesh. In contrast to raw apple tissue, representing a very heterogeneous material from the structural and rheological points of view, cells of potato tissue are in perfect contact although some small intercellular voids exist. The intercellular volumes in potatoes are estimated at 1% of the total volume in the potatoes and are insignificant, while they are at 20–25% in apple (Aguilera & Stanley, 1990). It has been shown; that the intercellular space morphology affects mechanical properties of apple tissue (Khan & Vincent, 1993) and further the close arrangement of polyhedral potato cells endows the textural properties such as stiffness and crispness of potato tuber tissue. The sharp drop of E detected for the warm air dried apple cubes after 5 days of storage is mainly attributed to softening of the crispy outer crust due to the redistribution of water but not due to water absorption as no differences in dry matter content were measureable (data not shown). Similar effects were detected in freeze dried apple and potato cubes whereby textural properties did not change during storage. Variations in textural properties caused by different drying technologies are well known and highly dependent on product properties, process conditions and pretreatment of the fruits and vegetables used (Krokida, Kiranoudis, & Maroulis, 1999; Ramos et al., 2013). Depending on the degree of process intensity, cell disintegration index was slightly increased to a maximum of 0.12 (potato) and 0.16 (apple) and, consequently, must be taken into consideration as a possible reason for the textural changes obtained. In contrast to Tappi et al. (2014), who attributed the detected modifications of linear distance and gradient during texture analysis of apple tissue to micro-structural alteration caused by a sort of bio-film, probably generated by the destruction of superficial cells promoted by gas-plasma oxidant radicals, the treated samples seemed covered by, from a visual examination no effects on the surface properties of the apple and potato tuber tissue were observed in this study.

Effects on browning index of the tested material are most likely caused by differences in refraction as well as by enzymatic and non-enzymatic browning reactions. As the latter is favored by heat treatments including a wide number of reactions such as Maillard reaction, caramelization, chemical oxidation of phenols, and maderization (Tappi et al., 2014), warm air drying at 65 °C may have led to non-enzymatic browning reactions in this study. Upon storage for 19 days at 20 °C enzymatic browning is considered to be the most likely reason for the increase in browning index determined for warm air dried apple cubes. As color changes on the apple cubes' surfaces were visible immediately following plasma treatment and PPO and POD activities were demonstrably reduced, reactions causing the occurring effect on product color must have been caused during plasma treatment. One possible explanation might be given by non-enzymatic reactions of secondary plant metabolites triggered by plasma-immanent species (Grzegorzewski et al., 2010). Plasma-oxidative degradation or polymerization of components contained in apple flesh may have led to the product-specific discoloration.

5. Conclusion

The findings of this study underline the potential of PPA processes in the field of food processing as it has been shown that PPA is capable of reducing the activity of the quality-determining enzymes PPO and POD in fresh cut and dried apple and potato tissues. The variation of treatment time and drying technology applied demonstrate that the process conditions have a significant impact on the success of enzyme inactivation and product quality parameters, whereby the product-specific optimization of treatment parameters remains a challenge. Further the results regarding color and texture of this study underline the specificity of plasma-induced effects on the product quality. As consumers take product appearance into consideration as a primary criterion; browning of the apple tissue constitutes a disadvantage. In contrast, the application of PPA to potato improved the product quality by causing a more natural color impression of the freeze dried potato cubes while achieving complete prevention of blackening. Combining its non-thermal character and its ability to inactivate both enzymes and microorganisms, PPA could be an alternative to traditional processes, as the promising results and the advantages of PPA (low-temperature, penetration of gaps, simple and cheap generation) provide a wide range of possible applications in the food sector. With the appropriate selection of raw materials, the application of PPA processing may offer an operation of improved sustainable strategies for reducing losses and providing high quality and safe commodities in the minimal processing industry for fruit and vegetables. In this context the results of this study indicate the possibility of integrating the PPA technology as a

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pre-drying procedure into existing process chains of selected commodities.

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5. Conclusion and perspectives

Several possible applications along the entire value chains of various foodstuffs can be derived from the results and findings of this work (Fig. 23). These may be divided into the production of high- and low-moisture plant and animal based products providing different initial product properties and thus, demanding for individual plasma processing conditions in order to appropriately achieve the defined process targets. With regard to the integration of the CAPP technology into the value-added chains of high-moisture produce of plant and animal origin during the early stage of cultivation and rearing/breeding, the plasma treatment of pea seeds, sprouts and juvenile plant, as well as the exposure of larvae from edible insects to plasma were found to be promising.

Results of the present work on the application of CAPP to pea seeds, sprouts, and seedlings during growing evidently showed that the plasma-immanent species are capable of stimulating changes in germination and growth rate of the seeds followed by modifications of physiological fitness and synthesis of valuable flavonol gylcosides in sprouts and juvenile plants. Pea seed germination rate was increased with duration of plasma exposure and was followed by reduced growth rates and increased dry matter contents of evolved sprouts. It was further demonstrated that freshly germinated grain peas respond to exposure to CAPP produced from ambient air, whereas the response of photosynthetic efficiency of seedlings and sprouts greatly depended on the growth stage of the peas, at which the treatment took place, and on its duration. Nevertheless, all treatments tested decreased the photosynthetic efficiency of seedlings, which is assumed to be related to the doses of ROS and UV radiation based on the plasma parameters selected. Exposure to CAPP modified the flavonol glycoside profile in pea seedlings. For short exposure times, the dose-dependent decrease in flavonoid contents was found to be most likely attributed to high concentrations of UV-C, which did not act as a constant trigger for enhanced flavonoid biosynthesis, and has to be considered as

i. a consequence of the impact on photosynthetic efficiency and on related signaling pathway(s) involved in the synthesis of plant secondary metabolites,
ii. the protection against oxidative stress resulting from excessive strain by ROS and UV radiation from CAPP,
iii. as well as of the chemical modification of the target molecules, which may be induced by plasma-immanent species.

Further investigations should include the analysis of plasma-initiated reactions and the effect of subsequent low doses of CAPP to gain deeper knowledge in plasma-dose-response-relationships for the production of a health-promoting functional food. Up to now, the CAPP application to seeds, growing sprouts and seedlings during rearing seems to be most promising by repeated interval semi-direct exposure to DBD-generated CAPP or by indirect exposure to plasma using PPA produced via a MW driven plasma source and inserted into appropriate growth chambers. Both plasma treatment concepts are also conceivable as a gentle decontamination method of insect larvae in order to keep the microbial load on the

larvae surface to a minimum throughout rearing and thus, producing microbial safe fresh insects.

During post-harvest processing of plant and animal based foodstuffs the production or modification of respective valuable ingredients in liquid form represents another promising approach of the CAPP technology, which may also be of importance in related scientific fields. Important basic indications for this assumption are also provided by results and findings obtained in this thesis, as the basic hypothesis – the plasma-induced formation of nitrated phenolic compounds - could be evidenced, as mono-nitrated reaction products were identified by their specific m/z value for all three focal phenolic model substances tested.

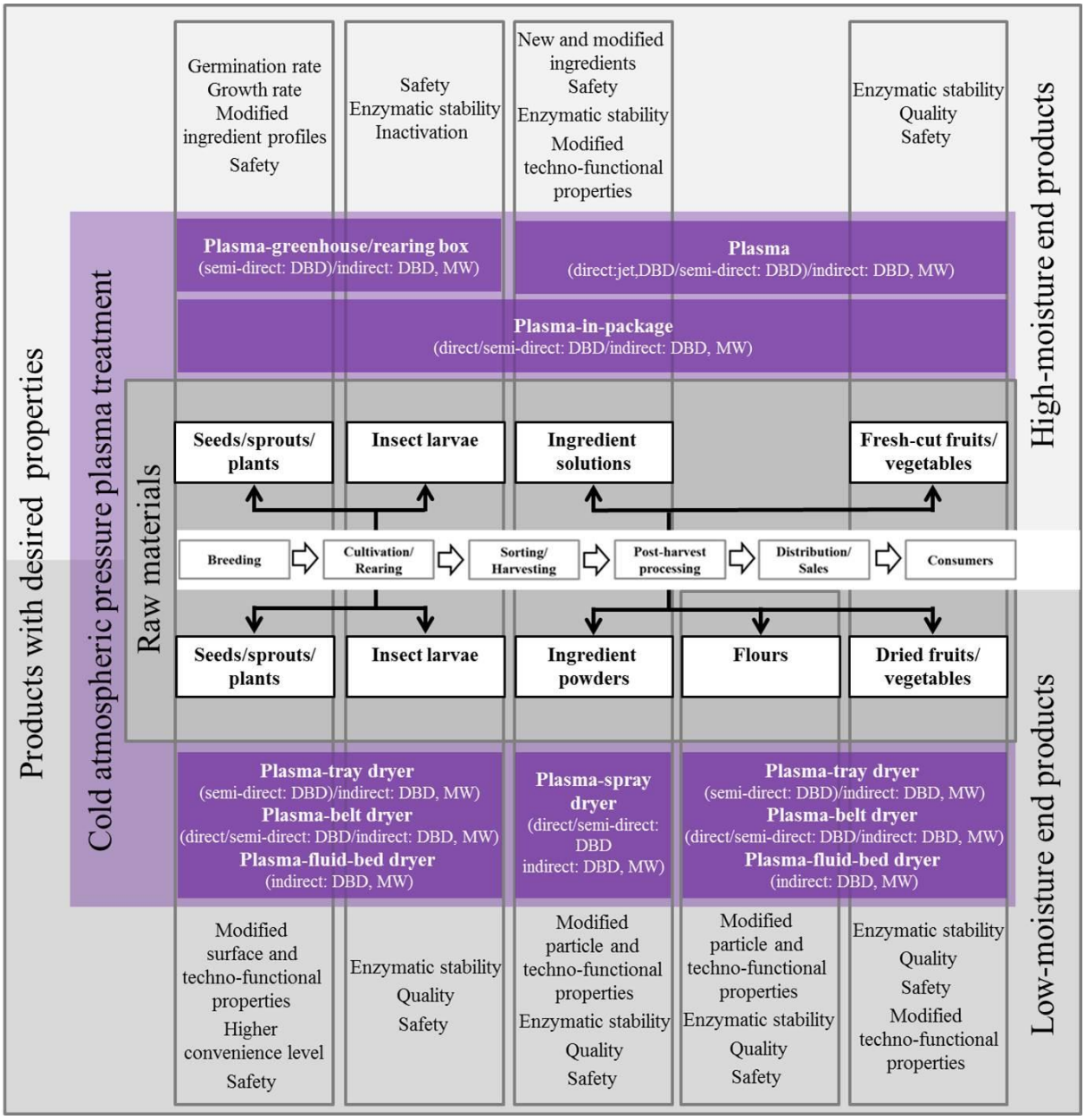

Figure 23: Possible CAPP applications along value-added chains of various foodstuffs derived from the results and findings of this work divided into the production of high- and low-moisture plant and animal based produce.

Nitration reactions, formation and degradation of reactants were found to be dependent on exposure time to PPA. The main reactions observed, when the liquid model phenolics

(pyrocatechol), as well as characterized derivatives of hydroxycinnimic acid (chlorogenic acid and caffeic acid) were exposed to PPA, were attributed to oxidation, nitration and polymerization of the phenolic compounds, but underlying reaction mechanism could up to now not be fully identified. Comparable CAPP-induced nitration and nitrosation reactions with aromatic molecules were also detectable following PPA treatment of solutions containing selected aromatic (tryptophan, tyrosine) and non-aromatic (lysine) amino acids (Bußler, unpublished data), which were accompanied by the typical yellow coloring of the solutions already described in a study conducted by Segat *et al.* [116]. The reactions identified were found to be specific for and limited to aromatic amino acids and could up to now not be evidenced in case of treating solutions of complex globular model proteins (bovine serum albumin (BSA), beta-lactoglobulin (β-LG)). The application of CAPP lead to the formation of turbidity in the protein solutions, which was more pronounced in case of BSA (A2.1, see annex II). For both focal model proteins, neither the presumed formation of stable aggregates could be evidenced during electrophoretic separation via SDS-Page non-reducing conditions (A2.2), nor an impact on their composition. Solubility was reduced in case of β-LG, but no clear correlation between change in solubility and exposure time was found (A2.3). In contrast, a clear correlation between protein solubility and exposure time was found in case of BSA, as it was linearly decreased with increasing treatment time. Maximum tryptophan fluorescence intensities (TFI_{max}) were detected at 334 nm (λ_{max}) in case of β-LG and at 348 nm in case of BSA, whereas no shift in λ_{max} was measured upon extended exposure to CAPP (A2.4). Taking into account the residual protein concentrations, TFI_{max} linearly decreased with extended exposure to CAPP in BSA solution, whereas no clear correlation was found in case of β-LG. Protein-specific changes in surface hydrophobicity index (SHI), which again could not be clearly correlated to treatment time, suggest that protein-specific plasma-induced unfolding reactions resulting in modified secondary and tertiary structure of the proteins may be the reason. Circular dichroism (CD) spectroscopy evidenced protein-specific unfolding, as changes in shares of α-helix, β-sheet, turn and unfolded fractions in both model protein solutions showed up (A2.5).

Although the underlying mechanisms are not fully elucidated, the results obtained clearly demonstrate

i. the occurrence of protein-specific plasma-induced modifications down to the secondary structure level probably caused by
ii. plasma-induced photochemical, redox, fragmentation and cross-linking reactions, as well as by the modification of functional protein groups, which in perspective of their controllability via variations of the process parameters may be utilized for
iii. the targeted modification of techno-functional protein properties, protein digestibility, and even protein-associated allergenicity [193].

As end-products, it would be most practicable to plasma-treat the solutions in-package. Further, a plasma-treatment immediately prior to incorporation of the produced/modified ingredients into end-products, for instance in order to use short-lived effects, as enhanced surface stabilizing properties for producing foams or emulsions with plasma-modified

proteins, is also conceivable.

Parts of the thesis further evidenced the transferability of plasma-induced modifications of protein structures to enzymes contained in complex food matrices, as it was found that the CAPP technology is capable of reducing the activity of the quality-determining enzymes PPO and POD in fresh-cut apple and potato tissue. As the application of identical process conditions led to different results regarding the desired enzyme inactivation and quality parameters obtained upon variation of the raw materials treated, the results further underline the product-specificity of the plasma-induced effects. Whereas the modulus of elasticity of apple cubes was not significantly affected by exposure to PPA, it was significantly reduced with regard to potato tuber tissue. Also in case of color changes, product specific effects became apparent upon PPA treatment. While exposure to PPA did not affect the browning index of freshly cut potato tuber cubes, it was increased for freshly cut apple cubes - the apple tissue surface appeared brown. Extending the exposure to PPA did not intensify the surface discoloration, but increased the visual penetration depth of PPA-induced browning reactions into the apple tissue. As the color changes on the apple cubes' surfaces were visible immediately following PPA treatment and, at the same time, PPO and POD activities were demonstrably reduced, reactions responsible for the occurring discoloration must have been caused during exposure to plasma. One possible explanation might be given by non-enzymatic reactions of secondary plant metabolites triggered by plasma-immanent species. Plasma-oxidative degradation or polymerization of components contained in apple tissue and cell juice released from cells destroyed by cutting and adherent to the cutting surfaces may have induced the product-specific discoloration. From marketing point of view, browning of the apple tissue constitutes a disadvantage, since consumers take product appearance into consideration as a primary criterion. In contrast, the application of PPA to potato seemed to improve the product quality by causing a more natural color impression while achieving complete prevention of blackening. Thus, with particular attention to keeping the product quality, the product-specific optimization of treatment parameters remains a challenge. Combining its nonthermal character and its ability to inactivate enzymes and, if desired, microorganisms, PPA processing with its advantages (low-temperature, penetration of gaps, simple and cheap generation) could be an alternative to traditional processes in the production of fresh-cut produce. However, three further important aspects have also come to the fore, which will significantly contribute to identifying new priority areas of future scientific issues:

i. PPA-induced browning reactions must have been of non-enzymatic character and may be due to interactions with secondary plant metabolites, as evidenced in phenolic model solutions and plant material from peas.

ii. Hence, oxidation and polymerization of phenolic compounds must be taken into consideration as a possible cause for the PPA-induced discoloration of the apple tissue.

iii. Besides the formerly mentioned probably induced reactions, which are mainly quality-determining, preventing the formation of hazardous nitro-phenolic compounds in complex food matrices upon exposure to PPA must be ensured in any case.

Nonetheless, with detailed knowledge regarding the plasma-specific reactions, the appropriate selection of raw materials and process parameters, the application of PPA processing may offer an operation of improved sustainable strategies for reducing losses and providing high quality and safe commodities in the minimal processing industry for fruit and vegetables. In this context, the results of this study indicate the possibility of integrating the PPA technology as a final or in-package application into existing process chains of selected fresh-cut commodities.

With regard to the integration of the CAPP technology into the value-added chains of low-moisture produce of plant and animal origin during the early stage of cultivation and rearing/breeding, again, the treatment of pea seeds, sprouts and juvenile plants, as well as the exposure of larvae from edible insects to CAPP is considerable in case drying is the subsequent unit operation to obtain low-moisture products. In case of producing dried seeds, the plasma-induced seed and hull surface modifications, which were evidenced in parts of this thesis, may lead to accelerated water uptake during soaking, increased germination ability and improved cooking properties during further processing or preparation by the consumer. This could contribute to increased convenience and thus, growing consumer acceptance among so far unpopular products, which are further difficult to market. In case of producing dried edible insect larvae, treatment with CAPP could successfully contribute to increase their final quality and safety attributes, as an increase in stability by enzyme inactivation and subsequent prevention of undesired browning reactions during drying, as well as an inactivation of microorganisms - at least on the surface of the insect larvae - can be achieved. In both cases, the integration of the CAPP technology into the value-added chains would be particularly promising by combining an indirect, semi-direct or direct plasma treatment with drying and, thus implementing a plasma-assisted drying step, which could be realizable by

i. feeding tray, belt or fluid bed dryer with PPA generated via a DBD or MW driven plasma torch coupled to the dryer from outside (indirect) or by

ii. implementing DBD plasma sources inside a tray, belt or fluid bed dryer enabling either the direct plasma application by passing the products to be dried over the plasma sources or the semi-direct application by mounting the plasma sources on the dryer ceiling directed towards the products to be dried.

With respect to this possible future application of CAPP, first results of studies regarding plasma-product interactions investigating the induced mass losses, average surface temperatures and the resulting plasma composition occurring upon direct CAPP treatment (plasma jet kINPen©) of solid model food systems with varying a_w-values (Bußler, unpublished data) under variation of the process gas used for plasma ignition, are presented below (Fig. 24 to 26). The results represent first experimental steps towards investigating the combinability of CAPP and drying technologies to one unit operation.

Direct CAPP treatment of the solid model food systems induced significant mass losses (Fig. 24), which are attributed to the evaporation of water and, due to the current flow of the working gas, also occur without igniting the plasma. Although the experiments did not aim at accelerating the removal of water, as this will proceed by the dryer in prospective plasma-

assisted drying, the plasma-induced "drying speed" was dependent on the process gas used and further was found to be increased compared to not ignited plasma for all working gases used.

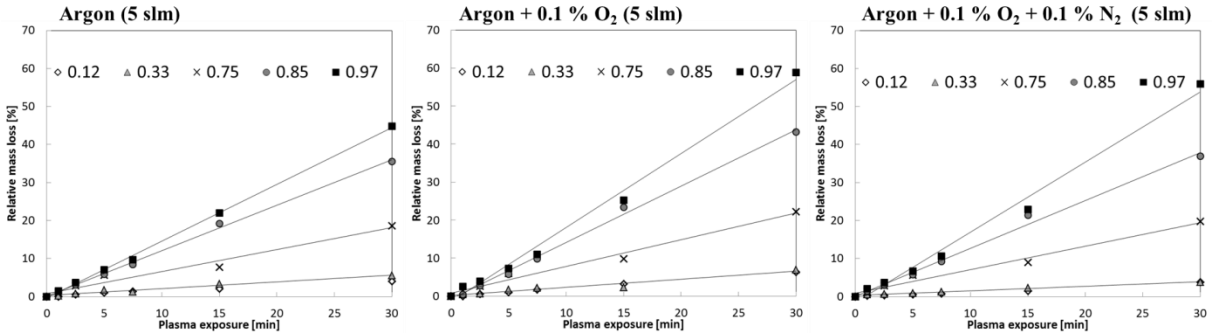

Figure24: Time dependent mass loss of solid model systems with varying a_w-values exposed to direct CAPP treatment (plasma jet kINPen©) under variation of the working gas (Bußler, unpublished data).

Depending on feed gas composition, higher a_w-values and concomitant larger amounts of evaporating water lead to cooling of the sample surface, lower water availabilities lead to increased surface temperatures of up to 35 °C (Fig. 25).

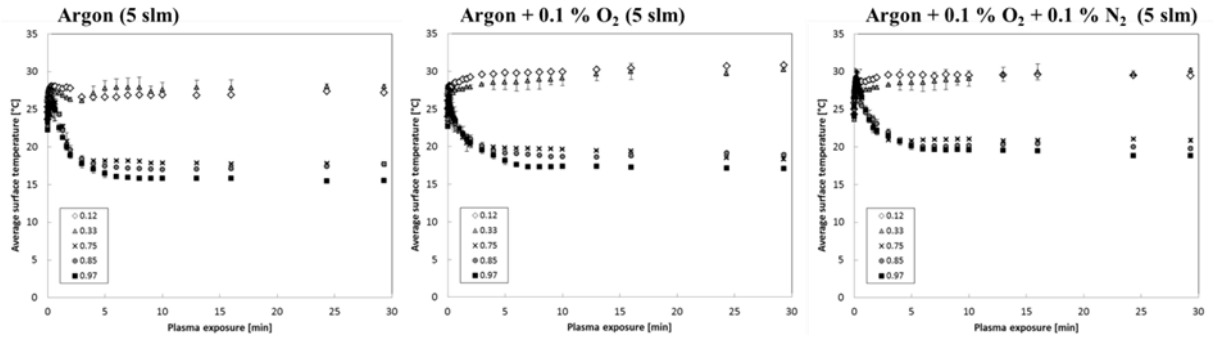

Figure 25: Time dependent average surface temperature of solid model systems with varying a_w-values exposed to direct CAPP treatment (plasma jet kINPen©) under variation of the working gas (Bußler, unpublished data).

In turn, the composition of the plasma applied was affected by the aw-value of the solid model system treated, the exposure time applied, and the working gas used (Fig. 26). The product and process parameters significantly influenced the presence of certain plasma-immanent species in the afterglow of the plasma jet (for more figures and details on materials and methods see Annex III, A3.1 to A3.3).

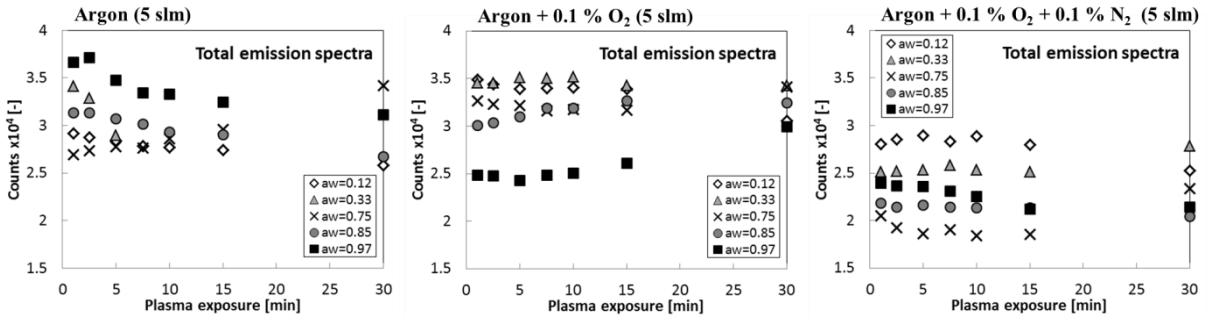

Figure 26: Area of the total emission spectra of the plasma afterglow (plasma jet kINPen©) during exposure to solid model systems with varying a_w-values under variation of the working gas measured via optical emission spectroscopy (Bußler, unpublished data).

Also using these simple model systems, the product-process interactions during CAPP application were found to be manifold and strongly dependent on product properties and process parameters. Specific knowledge on appropriate interactions is necessary to control the impact on the treated matrix and, hence, must be taken into consideration for developing tailor-made process designs.

In post-harvest processing of plant and animal based foodstuffs, plasma-spray drying could further offer an innovative approach during the production or modification of respective valuable ingredients in powder form. If the plasma-induced modification and production of substances in liquid systems, as evidenced in parts of this work, also proceed during simultaneous spray drying, indirect, semi-direct and direct plasma-spray drying would be feasible by

i. feeding the spray dryer with PPA, comparable to possible tray, belt or fluid bed plasma-dryers, by
ii. implementing DBD plasma sources inside the drying chamber enabling the semi-direct application, or by
iii. developing a plasma-jet system for the direct application of CAPP by spraying both, the liquid and the plasma, directly out of the nozzle of the jet.

Besides contributing to improved safety and quality attributed of the powders produced, which could be achieved by the effective inactivation of contained undesired quality-determining enzymes and potentially pathogenic microorganisms, plasma-spray drying could further induce desirable modifications of particle properties, as particle shape, particle size (distribution), fluidity and density and could, thus, lead to improved processability, packaging, and techno-functional properties of the powders.

Also with respect to this possible future application of CAPP, first results of studies regarding plasma-product interactions investigating the induced mass losses, average surface temperatures. and the resulting plasma composition occurring upon direct CAPP treatment of liquid model food systems with varying a_w-values (Bußler, unpublished data) under variation of the process gas used for plasma ignition, which represent first experimental steps towards investigating the combinability of CAPP and drying technologies to one unit operation, were conducted (for more figures and details on materials and methods see Annex III, A3.4 to A3.6). Findings were consistent with those revealed from experiments using solid model food systems with varying a_w-values and further also pointed out the promising approach regarding the plasma-drying of liquids.

In post-harvest processing the plasma-induced modification of low-moisture plant and animal based produce was identified as another approach aiming at the production of health-promoting functional foods by investigating the CAPP-induced effects on flours and flour fractions from peas and edible insects in this work. The detailed characterization of the plant-based starting materials indicated that pea seeds can be utilized to prepare protein-rich intermediates in order to be used in the production of foods. Pea flour (PF), protein-rich pea flour (PPF), and pea protein isolate (PPI) were found to be highly soluble at alkaline pH

values. PPI was found to also have a high solubility at pH 2. Air classification (PPF) and isoelectric precipitation (PPI) allowed the concentration of field pea protein by nearly 3- or 4-fold in comparison to PF. Furthermore, techno-functional properties varied among different flour fractions and were found to be not only dependent on protein content, but also on the selected processing steps conducted. When comparing PF with commercial pea flour (cPF) of equal protein content, large differences in water and fat binding properties indicated further potentials in technological processing for effectively manipulating the techno-functional properties of pea flour fractions. The results evidenced that it is advisable to prepare protein-enriched flour fractions, such as PPF, rather than preparing protein isolates. The combination of several physical separation methods, such as air classification with different thermal conditions or innovative nonthermal processes, may provide more suitable techniques for preparing modified protein-rich intermediates rather than extensive isolation procedures. Similar outcomes resulted from the characterization of different flour fractions produced from larvae of two different edible insect species, which indicated that *T. molitor* and *H. illucens* can be utilized to prepare protein-rich intermediates to be used in the production of food and feed. Despite the solubility characteristics, which were shown to be specific for the insect species used, required processing parameters seem to be similar to those of plant proteins enabling the utilization of traditional methods in protein processing. *Tenebrio* flour (T-F) was found to be highly soluble at alkaline pH values. The proteins contained in defatted *Tenebrio* flour (T-DF) and in the high-protein fraction (T-HPF) were also highly soluble at pH 2. Defatting and isoelectric precipitation increased the concentration of proteins in T-DF and T-HPF. In case of *H. illucens*, defatting of the flour was less effective and needs to be further optimized. These results further indicate that it is advisable to prepare fat-reduced and protein-rich fractions, as both, protein content and quality with regard to solubility were improved. Results regarding the process-induced impact on the characteristics of the insect flour fractions indicate that the techno-functional properties can be effectively manipulated, but further research is needed to identify specific tools for tailoring them. Also with respect to microbial safety issues, a combination of several physical separation methods or the application of different thermal treatments may be more suitable techniques, but high temperatures and prolonged treatments can reduce the nutritional quality of isolates and intermediates. Alkaline processing can further alter protein quality due to undesirable reactions. Regarding the optimization of process routes for the production of insect-based food and feed products, many issues need to be considered. The results obtained in this study clearly underline the importance of tailored process design and indicated that the application of innovative nonthermal technologies should also be considered.

At this point, the application of the CAPP technology as an alternative nonthermal modification treatment for different pea and insect flour fractions came up and was compared to the effects induced by thermal flour treatments. In this part of the thesis, it was evidently ascertained that plasma-induced modifications of biomaterials' surfaces are transferrable to dry bulk food matrices. Regarding the plasma-induced effects on techno-functional pea flour characteristics, the modifying properties of the CAPP technology were evidenced, whereas plasma-induced effects on flour techno-functionality were dependent upon exposure time and

composition of the matrix treated. Further experiments using a pea protein isolate (PPI) indicated that these effects can be attributed most likely to CAPP-induced structure modifications of the proteins contained, which was also supported by detected changes in tryptophan fluorescence spectra. Particularly concerning the modification of protein- and fiber-rich matrices CAPP could provide an alternative nonthermal approach in the processing of bulky and powdery materials. In case of *Tenebrio* flour, CAPP treatment led to a significant reduction of the overall microbial load, whereas equally long thermal treatments at 120 °C and 140 °C were found to completely inactivate the native microorganism flora. Both treatments affected the techno-functional properties of the *Tenebrio* flour and solubility, structure, and composition of the proteins contained therein, but to a different extent. It appears that protein composition of the samples may have influenced the functional properties. The changes observed regarding protein solubility and fluorescence can be attributed most likely to heat-induced protein unfolding and denaturation as well as to reactions between plasma-immanent species and functional protein groups at the surface of the *Tenebrio* flour and protein particles. Findings regarding the CAPP-induced impact on the characteristics of the *Tenebrio* flour and proteins therein indicated that the techno-functional properties can be effectively manipulated. Especially with regard to the distribution of amino acid profiles and bioavailability of indispensable amino acids, further studies are required addressing bioavailability issues. However, the results represent an important step towards the development of sustainable and microbiologically safe plasma-assisted postharvest processing technologies for edible insects.

Here again, the combination of CAPP treatment and drying, *e.g.* by developing a plasma-fluid-bed dryer, seems to be most promising regarding prospective plasma treatments in post-harvest processing of plant- and animal-based flours also with the aim to prevent undesired plasma-induced heating of the bulk materials. In contrast to detected temperature changes on wet product surfaces characterized by increases to up to 45 °C or even lower [22, 147], during exposure of the pea flour fractions to CAPP a continuous temperature increase from ambient temperature to a maximum of 59.8 °C occurred. In case of *Tenebrio* flour, the bulk temperature rose to 67 °C. This increase in bulk temperature can be attributed to the heating-up of the plasma source during plasma ignition and the resultant insufficient convectional heat dissipation due to the short distance between sample and surface of the plasma source during lab-scale experiments. Further, the low water content of the flours prevents the evaporation of water and, consequently, evaporative cooling of the flour surfaces. For moist intermediates, which are first ground and then dried, as probably insect larvae, applying CAPP in combination with drying could further reduce the thermal load (as, depending on the drying temperature applied, evaporation of water will sufficiently cool down the flour particle surface) and possibly enhance plasma-specific effects (as the plasma-immanent species are more reactive, when the CAPP application is already running from the beginning of the drying step with high moisture content of the flour). As in case of plasma-spray drying, plasma-fluid-bed drying to produce plant- and animal-based flours could improve safety and quality attributes and could further induce desirable modifications of particle and techno-functional properties of the flours.

In case of producing cut and dried produce from fruits and vegetables combining the CAPP treatment with tray, belt or fluid bed drying would be suitable, thus inducing higher enzymatic and microbial stability and possibly enhanced quality (color and texture) as well as techno-functionality (reconstitution).

Finally, the work of this thesis significantly contributes to identifying and analyzing plasma-induced product-process interactions with the overall goal to reveal potential applications of the CAPP technology along value-added chains of food by

i. deriving tailor-made applications of CAPP based on detailed investigations of individual plasma-induced effects, reactions and interactions examined and evidenced by using model systems,

ii. providing evidence for the possible utilization of plasma-induced effects on plant secondary metabolites in order to selectively modify ingredient profiles in plant tissues and for using plasma-induced surface and ingredient interactions as a tool for the modification of proteins' tertiary and secondary structure and, thus, their functionality in dry raw materials, intermediates and products along the value-added chain of alternative plant protein sources in a targeted manner,

iii. transferring knowledge on plasma-assisted modification of dry bulk materials to animal-based raw materials from edible insects taking into account flour and protein modification, as well as microbial decontamination representing two specific key issues in the value-added chain for the insect-based production of foodstuffs, and by

iv. delivering initial approaches regarding the CAPP treatment of complex food matrices by using a plasma device, which is promising for the application in industrial scale, and further representing an initial approach towards the development of innovative process combinations with focus on "plasma-drying" as a prospective future unit operation.

Combing the CAPP treatment with unit operations being anyway part of an established process line further contributes to reducing the expected costs of the CAPP application. Possible future plasma-setups designed for plasma-drying of liquid and solid food matrices being already discussed throughout this section of the thesis are presented in figure 27. Up to now, financial and time demands for CAPP treatments are very difficult to set. In this work, plasma-setups operating under atmospheric pressure conditions and with air as the working gas (DBD device and PPA), which up to now certainly offer the most cost-effective alternative compared to other devices using noble gases for the generation of plasma, were used for the CAPP application to larger goods. However, the setups used only enable the batch-wise treatment on a laboratory scale. For industrial application of CAPP continuous operation mode and/or the integration of the treatment into existing production lines, as combining the CAPP application with transportation, drying, coating, *etc.* of the intermediates and products to be treated, is required. This makes the calculation of cost and time demands not yet appropriate, although basically, the plasma technology and the plasma sources used throughout the work of this thesis seem promising for up-scaling to industrial cost-effective use. In many industries and its related CAPP applications, the plasma technology combines

low running costs with high process and work safety and further represents a particularly rational process. As detailed calculations must consider costs of an industrial unit, capacity, working hours, product costs, *etc.*, and most of the data are not yet available for CAPP applications in the food sector, the appropriate calculation of process cost must be part of future works.

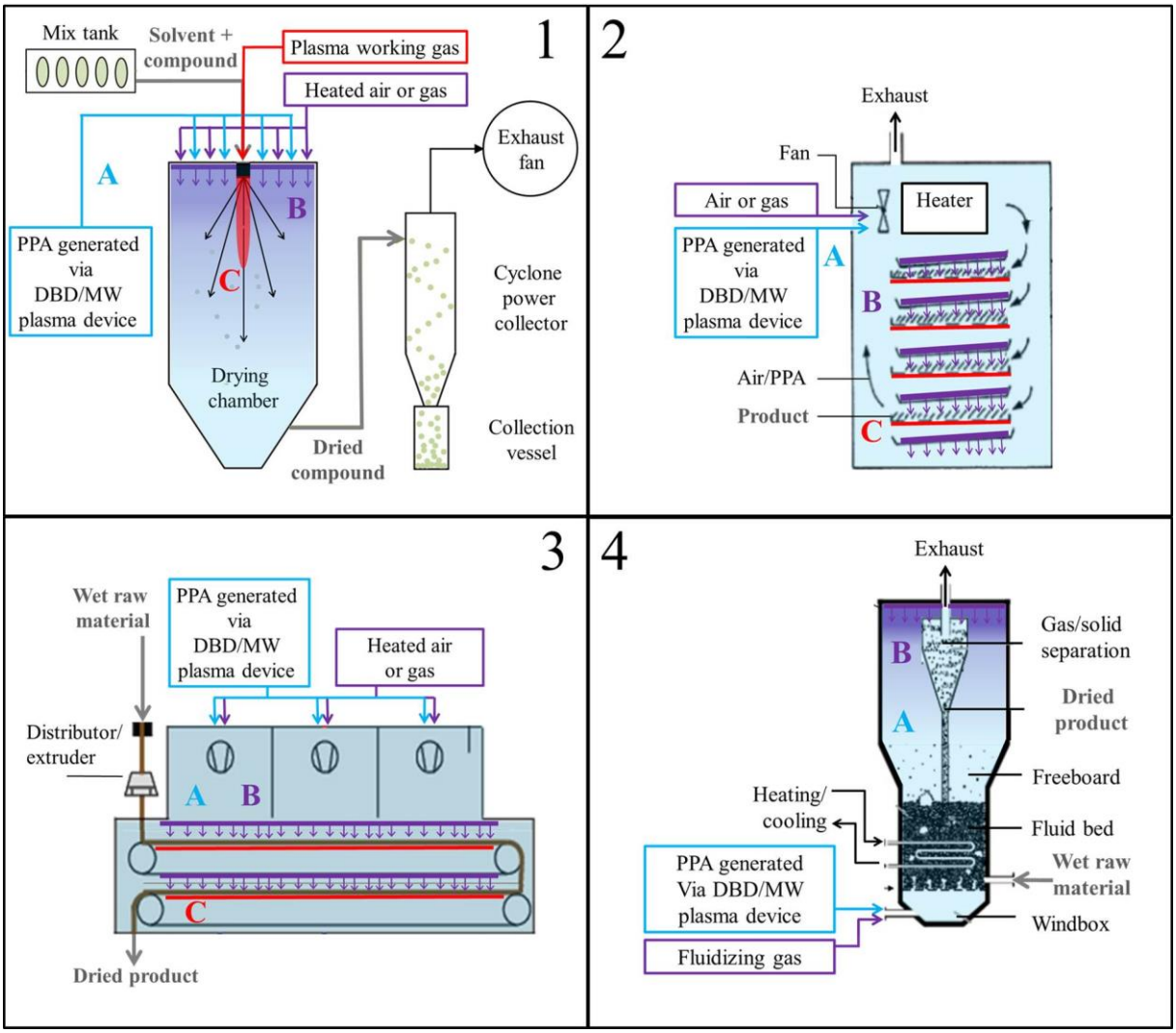

Figure 27: Prospective plasma-drying set-ups realizable by combinations of spray (1), tray (2), belt (3) and fluid bed (4) dryers coupled with plasma-jet, DBD or MW driven plasma devices for the indirect (A), semi-direct (B) and direct (C) CAPP application during drying of liquid and particulate raw materials, intermediates and products.

The development of innovative products and technologies enable innovations within the food sector, but not all technologies are equally accepted by consumers. Understanding and investigating consumer attitudes towards novel food processing technologies represent a specific key point for the developers of innovative food products and processes, as consumers may perceive the application of innovative technologies as more risky than using traditional food technologies. At present, the way wary consumers will react to cold plasma technology is unclear. On the one hand, the advertising industry could contribute to making the public aware of the benefits of the CAPP technology in food processing; on the other hand, it is the societal responsibility of scientists and engineers involved in the development of CAPP

technologies and food-related applications to share their knowledge with consumers in order to encourage their acceptance. A further challenge will be to achieve this within the confines of the regulatory requirements. Receiving approval for an innovative CAPP process with its complexity in plasma chemistry, the resulting large number of possible chemical effects, which need to be analyzed, prioritized, and assessed from the regulatory perspective, will require a significant amount of data and time.

The work conducted and findings obtained in this thesis underline the potential of the CAPP technology in the field of food processing. Due to its variety of application possibilities in the value-added chains of various food products, CAPP processing has the potential to become a routine tool for the food industry in the coming years. This is also reflected throughout all parts of this work, and thus, it provides a substantial contribution to promoting the successful admission of the CAPP technology in the food sector.

Finally, it becomes clear that detailed research studies from fundamental to applied level are needed in order to evaluate the safety of cold plasma-treated food products. Nonetheless, the future looks promising for implementing the CAPP technology in the food industry, as the opportunities for cold plasma technology in the food industry are only limited by the creativity of the inventor [194].

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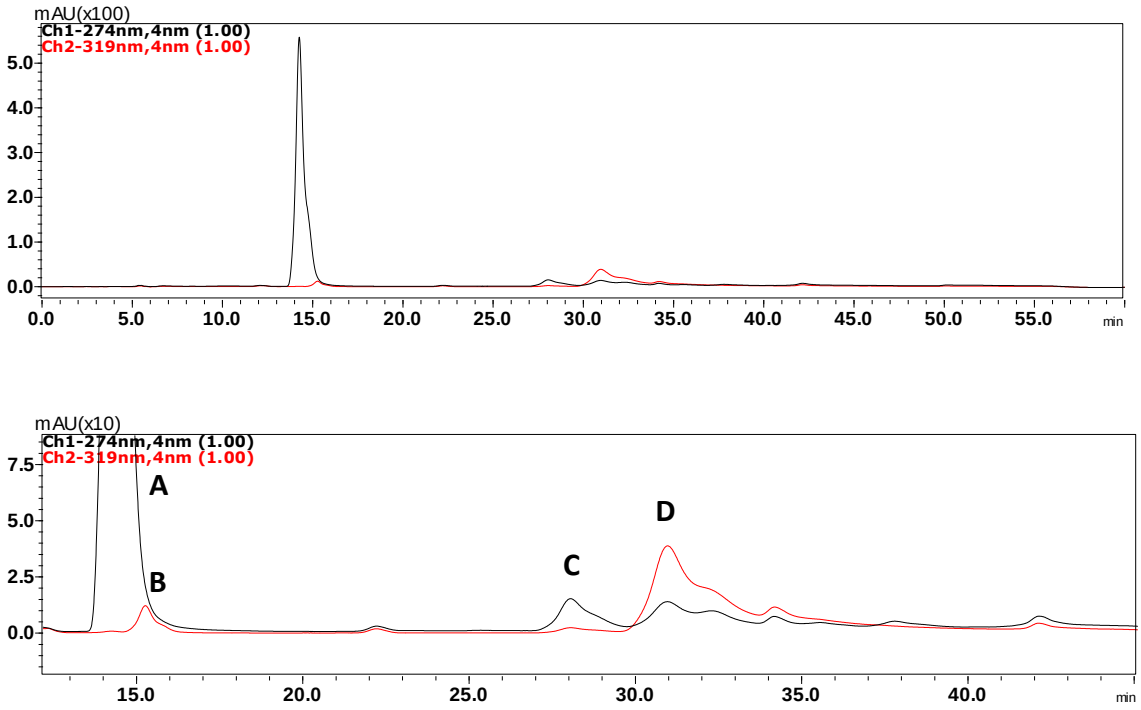
Annex I

Supplementary/Supporting Information: Section 3.2

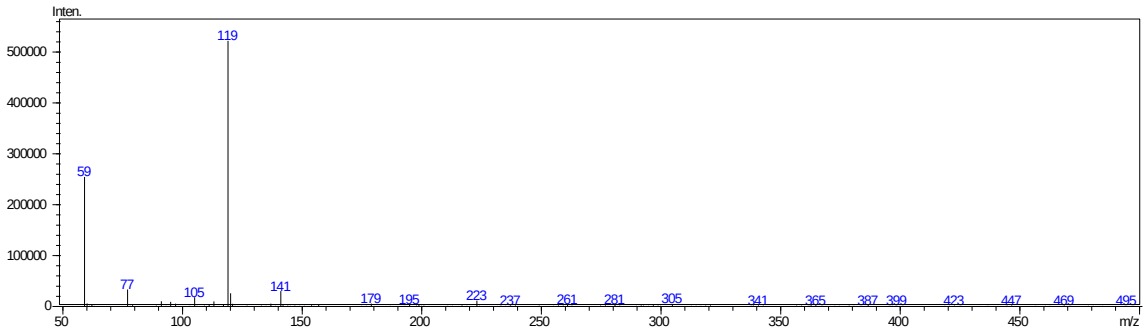
Determination of the reaction products via RP-HPLC/MS analysis

The method is described in the "material and methods section". In following some relevant chromatograms and mass spectra are illustrated.

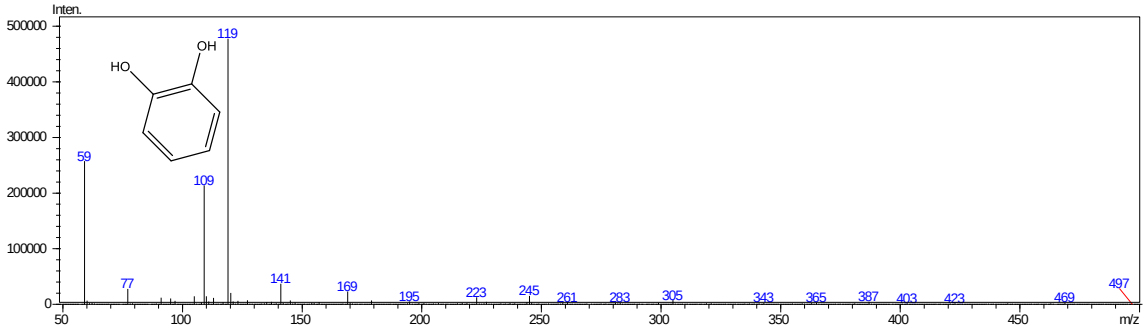
Catechol



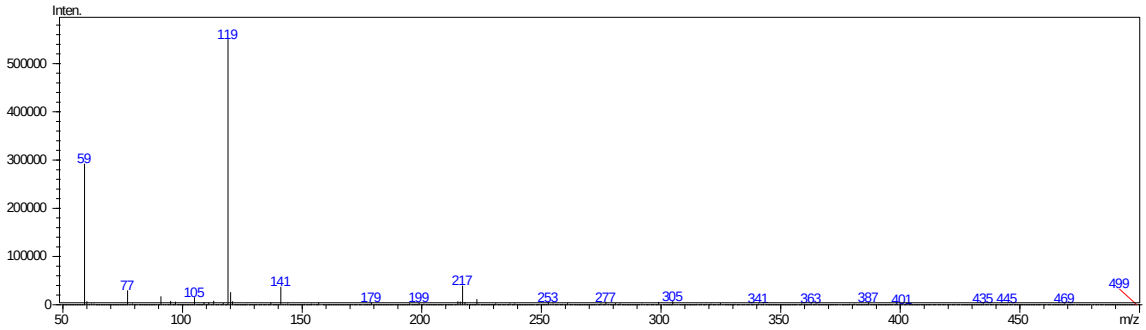
A1. 1: Exemplary RP-HPLC chromatogram of the separation of pyrocatechol after a 10 min exposure to PPA detected at 274 (black) and 319 nm (red) and plasma-induced adducts RPA, RPB and RPC detected. Code: A = Catechol; B = RP A; C = RP B; D = RP C.



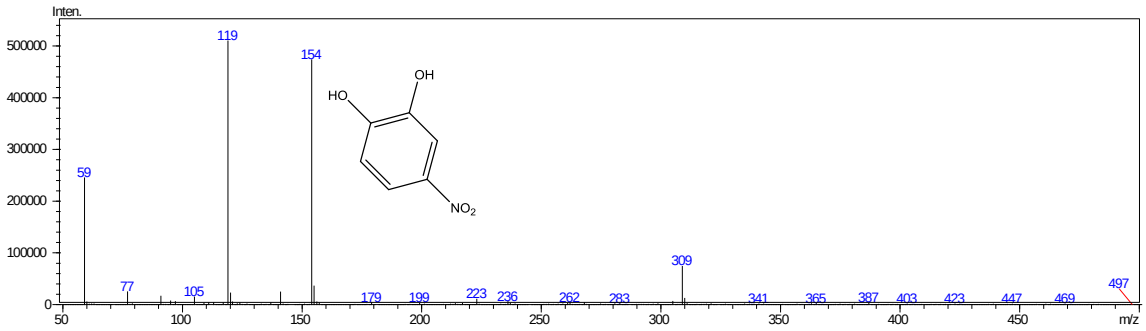
Eluent blank (Retention time = 10.017 min)



A = Catechol (Retention time = 14.433 min)



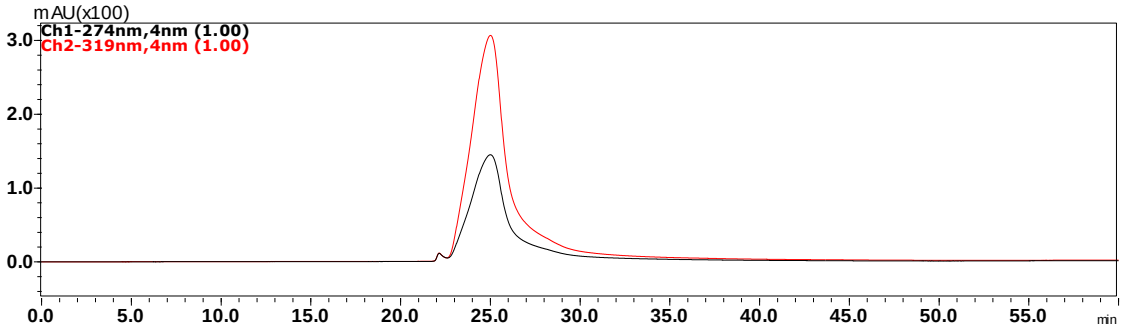
RP B (Retention time = 28.150 min)



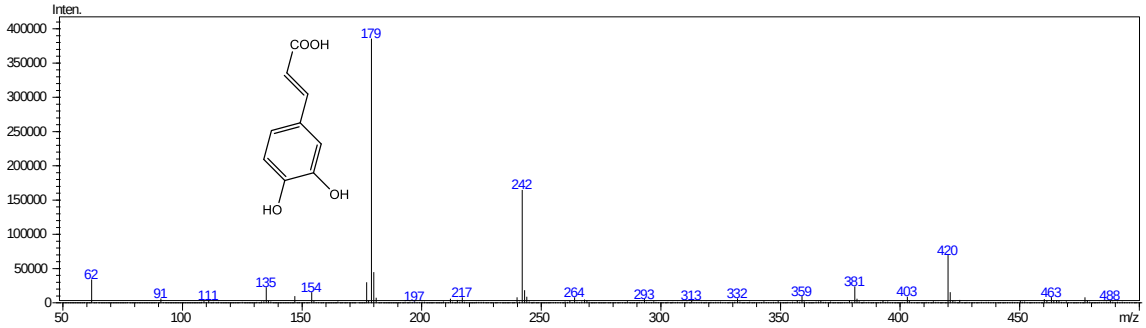
RP C (Retention time = 31.083 min)

A1. 2: The eluent (blank) itself showed two specific masses of 59/119 which could be not allocated but where present throughout the mass spectra. The prominent m/z values of the 10 min plasma treated catechol sample was: 109 at the retention time of catechol, none at the retention time of RP A (results not shown), 217 at the retention time of RP B (also reappearing repeatedly later on) and 154 & 309 at the retention time of RP C.

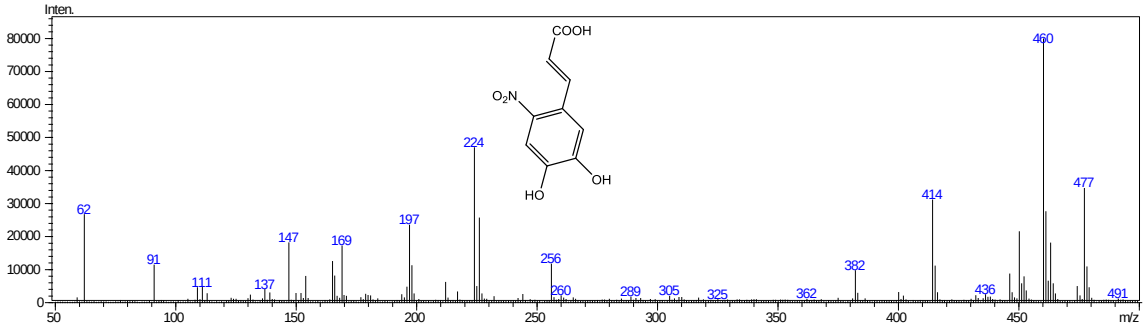
Caffeic acid



A1. 3: Exemplary RP-HPLC chromatogram of the separation of caffeic acid after a 10 min exposure to PPA detected at 274 (black) and 319 nm (red) and the plasma-induced adduct was also at the same retention time.

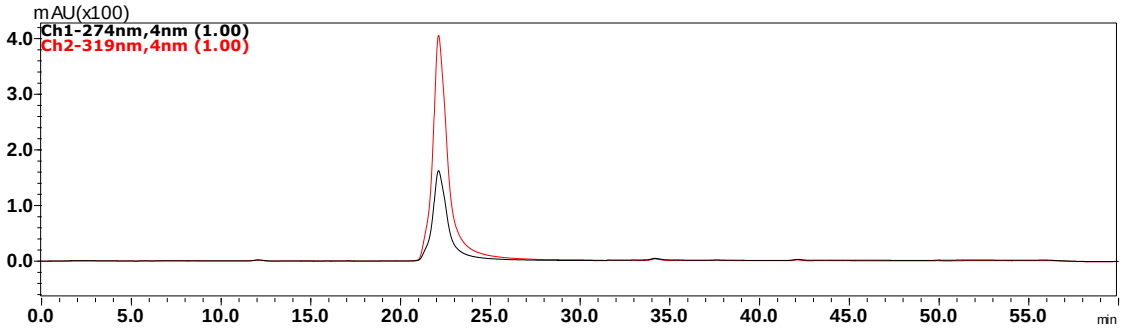


A1. 4: The prominent m/z values of the untreated caffeic acid sample were 179 and 242 (Retention time = 25.40 min).

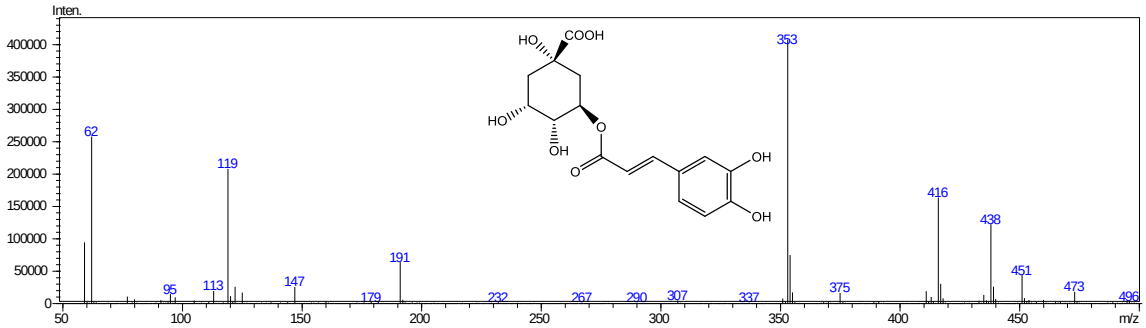


A1. 5: Plasma treated caffeic acid sample for the 10 min showed that these m/z values were barely detectable, instead the m/z value of 224 was prominent (Retention time = 25.883 min).

Chlorogenic acid - Untreated

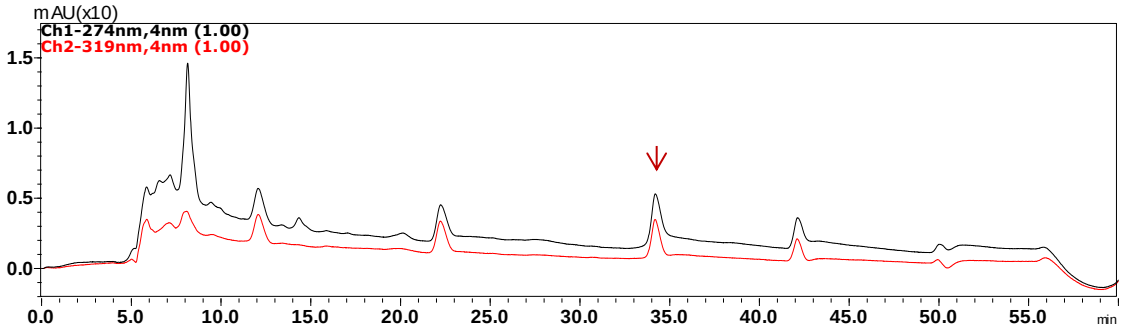


A1. 6: Exemplary RP-HPLC chromatogram of the separation of untreated chlorogenic acid detected at 274 (black) and 319 nm (red).

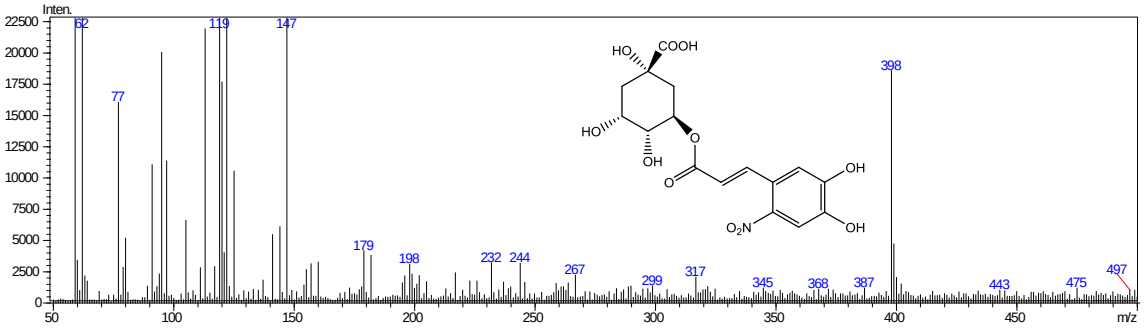


A1. 7: The most prominent m/z value of the untreated chlorogenic acid sample was 353 (Retention time = 22.217 min).

Chlorogenic acid – CAPP treated



A1. 8: Exemplary RP-HPLC chromatogram of the separation of chlorogenic acid after a 10 min exposure to PPA detected at 274 (black) and 319 nm (red).



A1. 9: For the 10 min plasma treated chlorogenic acid sample this m/z value was barely detectable. Instead, the m/z value of 398 (retention time = 34.367 min) was prominent.

Annex II

Supplementary/Supporting Information: Section 5

CAPP treatment of model protein solutions

CAPP treatment:
- plasma jet system (kINPen09©, INP Greifswald)
- feed gas: argon with 0.1 % oxygen; 5 slm)
- 2 mL model protein solution
- 2 cm distance to the plasma nozzle
- up to 10 min
- plasma-induced mass losses and impact on pH of the protein solutions were recorded
- separation of insoluble protein components by centrifugation (10000 g, 10 min, 4 °C)

Model protein solutions:
- 0.5 mg/mL of bovine serum albumin (BSA) and β-Lactoglobulin (β-LG) in 10 µM sodium fluoride phosphate buffer)

Protein solubility:
- Biuret assay (see section 4.1.3)

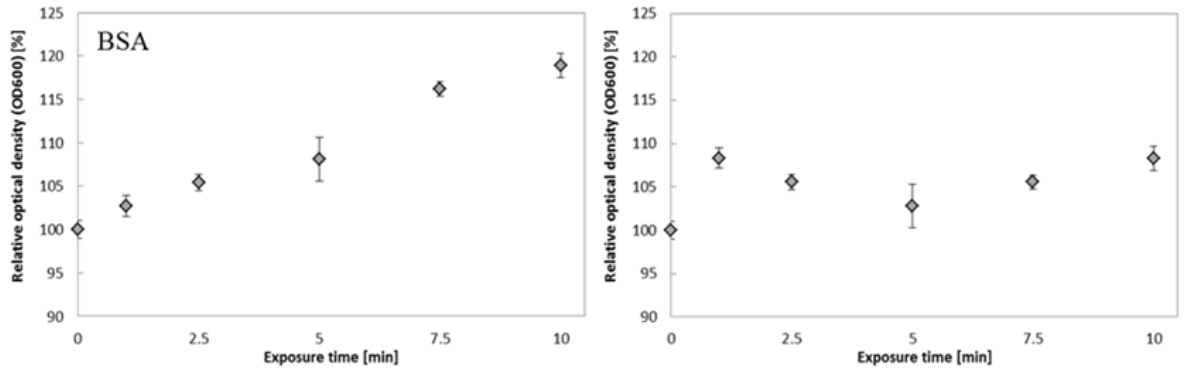
Optical density:
- OD 600 using an UV/Vis spectrophotometer (BioPhotometer plus, Eppendorf, Hamburg, Germany)

Tryptophan fluorescence:
- λ_{ex} = 290 nm, λ_{em} = 355 nm, peak height was analyzed (see section 4.1.3)

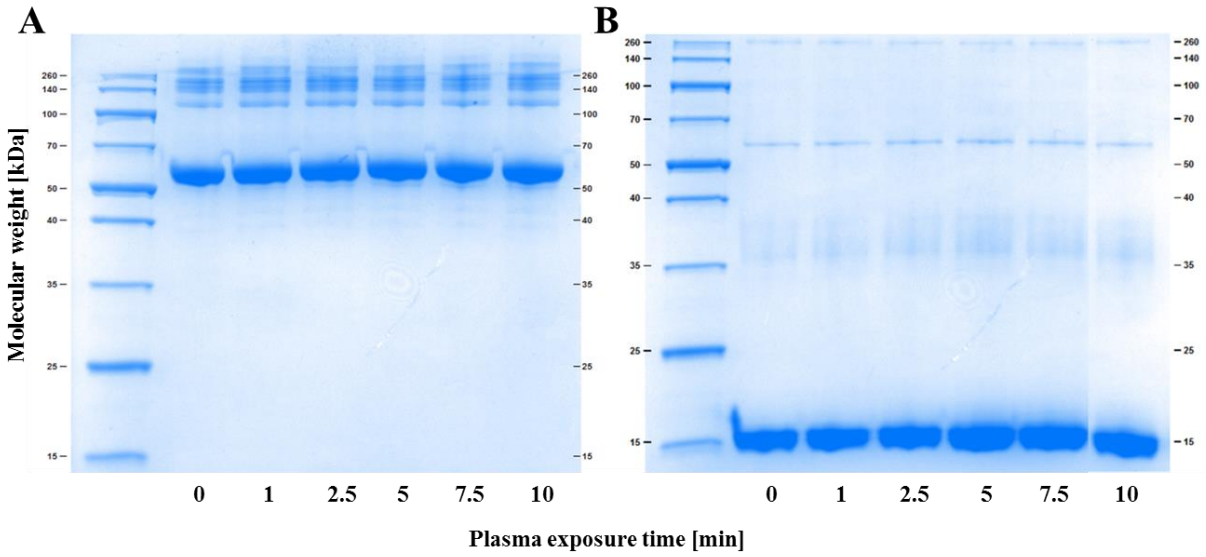
Surface hydrophobicity:
- 8-Anilinonaphthalene-1-sulfonic acid (ANS) method (see section 4.1.2)

Non-reducing SDS-Page:
- without addition of mercaptoethanol (see section 4.1.2)

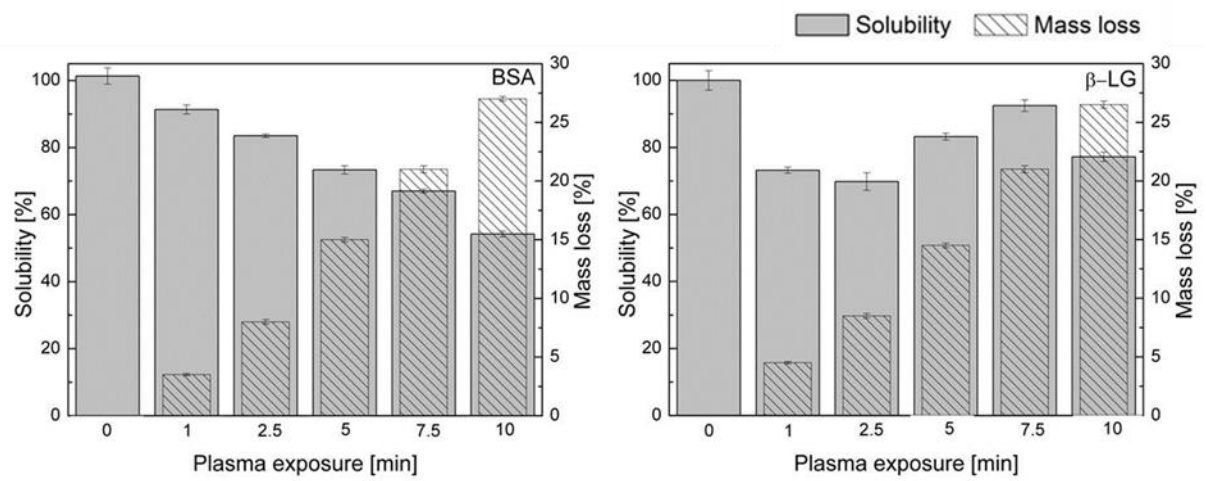
CD spectroscopy:
- sample dilution: with 100 mM natrium fluorid buffer (pH 7.4) to a final protein concentration of 0.5 µM,
- spectra record: Spectropolarimeter (J-710, Jasco Labor und Datentechnik GmbH, Großumstadt, Deutschland); exitation of peptide bonds: 180 – 240 nm: information on secondary structure on the protein and analyzed using
- spectra analysis: CD Pro SSE Analysis software (Jasco, SpectraManager). Plasma-induced effects on protein secondary structure were compared to those of a thermal treatment in a water bath (60 °C, 30 min)



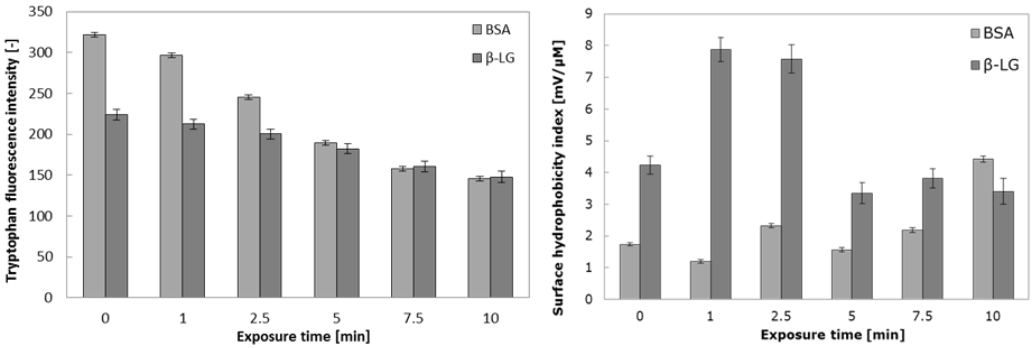
A2. 1: Plasma-induced impact on relative optical density of the model protein solutions BSA (Bovine serum albumin) and β-LG (Beta-lactoglobulin) at λ = 600 nm (Bußler, unpublished data).



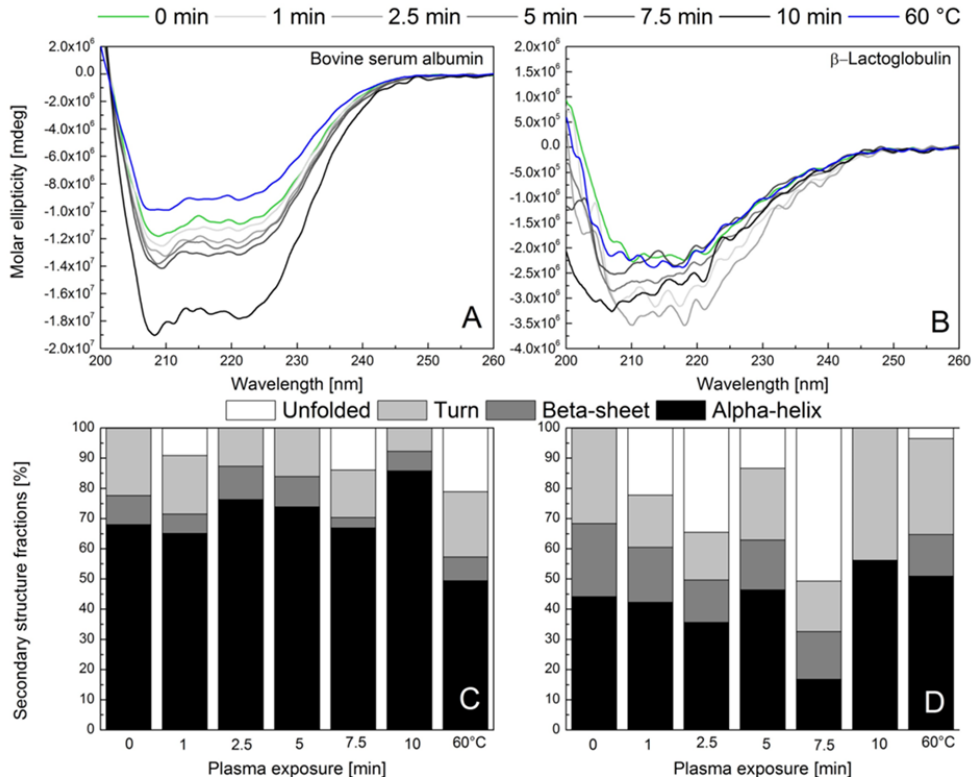
A2. 2: Electrophoretic separation of BSA (A, Bovine serum albumin) and β-LG (B, Beta-lactoglobulin) following exposure to direct CAPP treatment for up to 10 min.



A2. 3: Plasma-induced impact on protein solubility (pH 7.0, Biuret method - grey) and mass loss (hatched) of β-LG and BSA solutions (Bußler, unpublished data).



A2. 4: Plasma-induced impact on tryptophan fluorescence intensity (pH 7.0, λ_{ex} = 290 nm, λ_{em} = 334/348 nm – left) and on surface hydrophobicity (pH 7.0, λ_{ex} = 390 nm, λ_{em} = 470 nm - right) of β-LG and BSA solutions (Bußler, unpublished data).



A2. 5: Impact of plasma on secondary structure of BSA (A) and β-LG (B) depicted as change in molar ellipticity [mdeg] and plasma-induced change in secondary structure fractions in BSA (C) and β-LG (D) in comparison to thermal treatment at 60 °C. CD spectra recorded in the UV range between 200 and 250 nm (Bußler, unpublished data).

Annex III

Supplementary/Supporting Information: Section 5

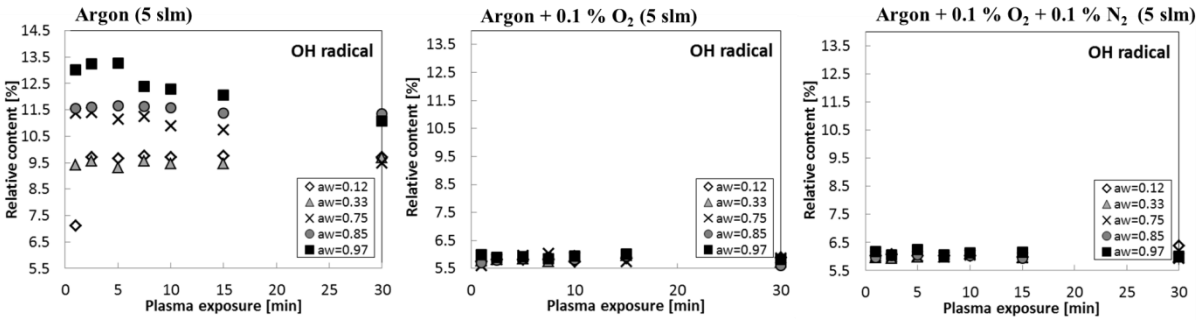
CAPP treatment of liquid and solid model systems

CAPP treatment:
- plasma jet system (kINPen09©, INP Greifswald)
- feed gas: argon, argon + 0.1 % oxygen, argon + 0.1 % oxygen + 0.1 % nitrogen; 5 slm
- 2 mL model solution / plate with agar gel
- 1.5 cm distance to the plasma nozzle
- up to 30 min
- plasma-induced mass losses and impact on pH of the protein solutions were recorded

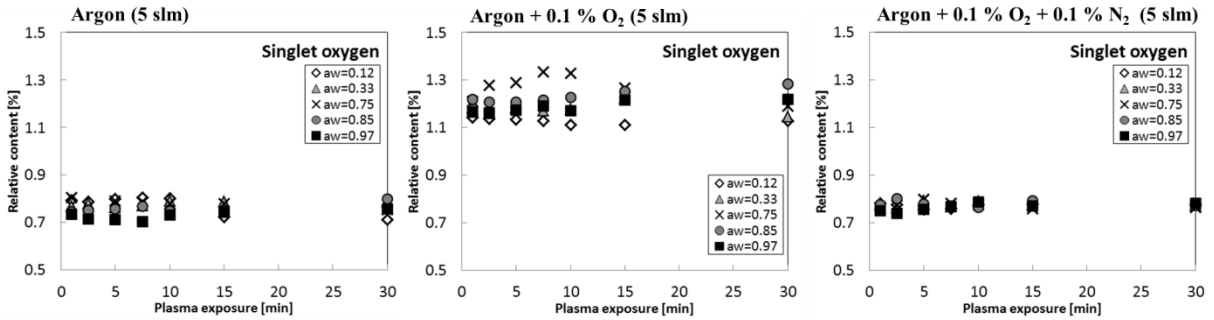
Model systems:
- liquid: water with the respective salt ($LiCl \cdot H_2O$ (a_w=0.12), $MgCl(H_2O)_6$ (a_w=0.33), NaCl (a_w=0.75), KCl (a_w=0.85) and K_2SO_4 (a_w=0.97))
- solid (agar gels) adjusted to the respective a_w-values with salt ($LiCl \cdot H_2O$ (a_w=0.12), $MgCl(H_2O)_6$ (a_w=0.33), NaCl (a_w=0.75), KCl (a_w=0.85) and K_2SO_4 (a_w=0.97))

Spectra record:
- UV–Vis Spectrometer (StellarNet Inc., Tampa, USA) equipped with a F400-UV–Vis-SR fiber optic

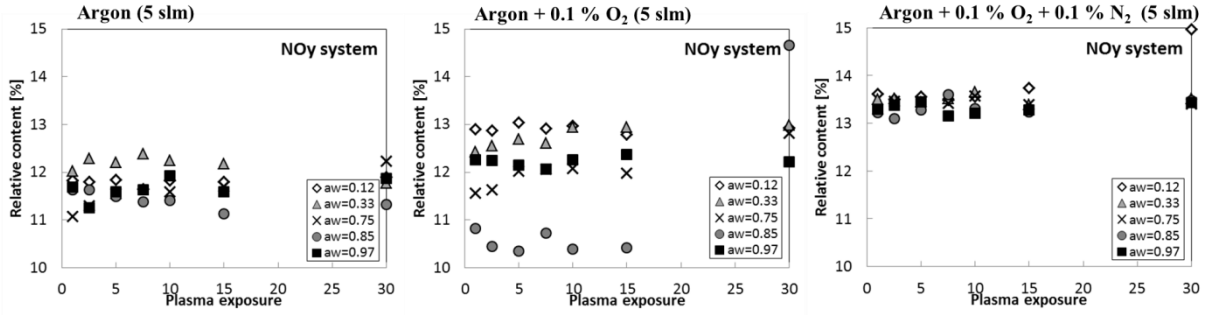
Average surface temperature:
- thermo-graphic camera



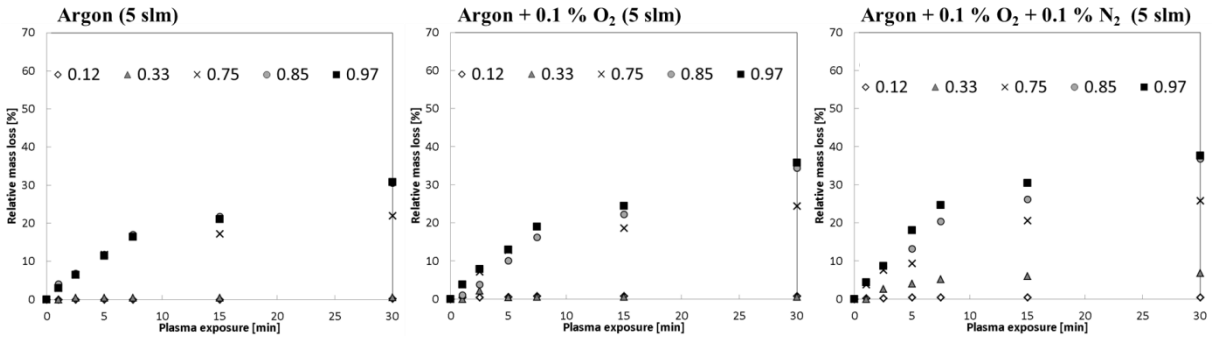
A3. 1: Relative OH content of the plasma afterglow during exposure to solid model systems with varying a_w-values under variation of the working gas measured via optical emission spectroscopy (Bußler, unpublished data).



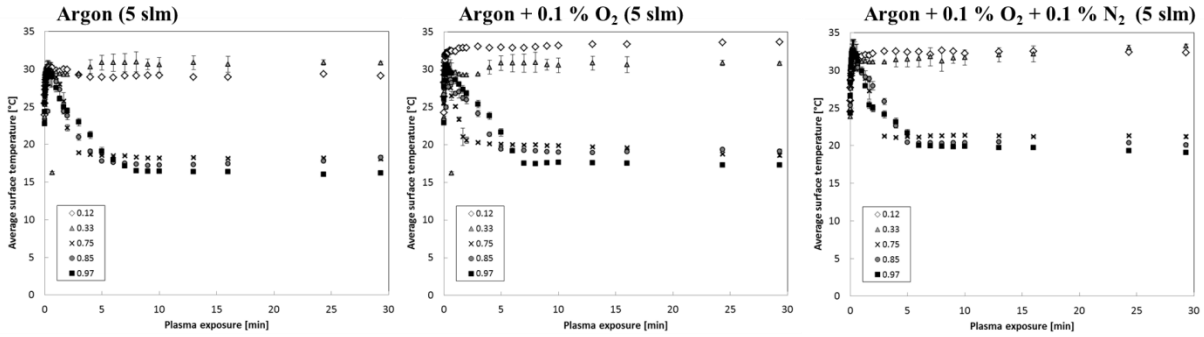
A3. 2: Relative singlet oxygen content of the plasma afterglow during exposure to solid model systems with varying a_w-values under variation of the working gas measured via optical emission spectroscopy (Bußler, unpublished data).



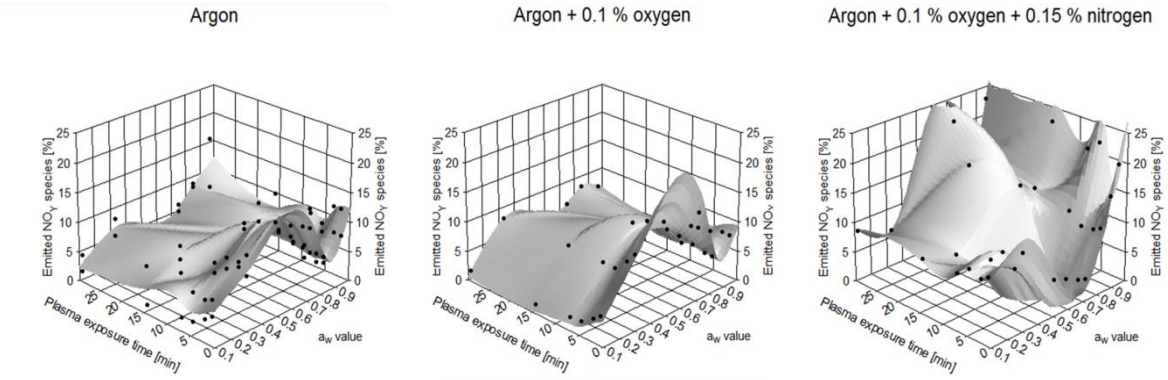
A3. 3: Relative NOy content of the plasma afterglow during exposure to solid model systems with varying a_w-values under variation of the working gas measured via optical emission spectroscopy (Bußler, unpublished data).



A3. 4: Time dependent mass loss of liquid model systems with varying a_w-values exposed to direct CAPP treatment under variation of the working gas (Bußler, unpublished data).



A3. 5: Time dependent average surface temperature of liquid model systems with varying a_w-values exposed to direct CAPP treatment under variation of the working gas (Bußler, unpublished data).



A3. 6: Emitted NO_Y species during direct plasma treatment as a function of exposure time and initial a_w-value (Bußler, unpublished data).

Curriculum vitae

Sara Bußler – Curriculum Vitae

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Main research fields

- Recovery of proteins from alternative sources as grain legumes and edible insects
- High pressure and cold atmospheric pressure plasma-induced modification of biomaterials in the food and feed sector
- Characterization of product-plasma interactions including safety aspects

Education

Since 02/2013	Leibniz Institute for Agricultural Engineering Potsdam-Bornim e.V. (ATB) Department of Horticultural Engineering Research program Quality and Safety of Food and Feed Research associate, PhD candidate
Since 11/2012	M. Eng. (Dipl.-Ing.) Food Technology Thesis: Processing of legumes – The influence of high isostatic pressure, pulsed electric fields and high temperature on the properties of pea flour and on yield and structure of soluble pea proteins
03/2012 – 11/2012	Technische Universität Berlin Student assistant in the High pressure research and application group, emphasis on high pressure modification of proteins
08/2011 – 07/2012	Technische Universität Berlin Student assistant with teaching responsibilities
10/2006 - 11/2012	Technische Universität Berlin Studies of Food Technology, emphasis on recovery of food ingredients

10/2005 - 09/2006	Freie Universität Berlin Studies of Biology

Research Projects

NutriAct - Nutritional Intervention for Healthy Aging: Food Patterns, Behavior and Products (FKZ 01EA1408F) Deutsches Zentrum für Luft- und Raumfahrt (DLR).

LeguAN - Innovative functional food and feed products based on regional grain legumes with special consideration of the whole food supply chain (FKZ 511-06.01-28-1-54.070-10) which is funded by the Federal Ministry of Food and Agriculture (BMEL) according to a decision of the German Bundestag.

Awards and Honors

11/2015	1st prize "Best Oral Presenter Award", 9th CIGR Section VI International Technical Symposium, 16-20 November 2015, Auckland, New Zealand (S. Bußler, L. Guérin, B. Rumpold, O. Schlüter, "An approach to optimize protein isolation from alternative sources like meal worms")
04/2015	Grant for the Training School of BacFoodNet (COST Action FA1202) on (Novel) physical intervention technologies for controlling food stability", 21-24 April 2015, Malta
11/2014	1st prize of the "GNT Young Scientist Award" presented at the Annual Meeting of the European Federation of Food Science & Technology (EFFoST) 2014, Uppsala, Sweden (S. Bußler, H. Rawel, J. Ehlbeck, O. Schlüter "Cold atmospheric pressure plasma: An innovative tool for the targeted modification of protein functionalities.")
05/2014	Poster competition, Honorable Mention at the International Food Congress – Novel Approaches in Food Industry - NAFI 2014, Kusadasi, Turkey (J. Jacob, G. Gündüz, S. Bußler, A. Fröhling, C. Theel, O. Schlüter "Comparison of the inactivation efficiency of two direct plasma treatments on model food systems")
11/2013	1st prize of the "Student of the Year Award" presented at the Annual Meeting of the European Federation of Food Science and Technology (EFFoST) 2013, Bologna, Italy (S. Bußler, "Processing of legumes – The influence of high isostatic pressure, pulsed electric fields and high temperature on the properties of pea flour and on yield and structure of soluble pea proteins")

Teaching activities

- Student assistant with teaching responsibilities (Technische Universität Berlin)
- Supervisor of 3 diploma/master and 1 bachelor thesis and 7 international interns

Reviewer Activities

- Food Research International

Membership in Scientific Organizations

- Institute of Food Technologists (IFT)
- International Association for Food Protection (IAFP)

Internship Experience

12/2012	Leibniz Institute for Agricultural Engineering Potsdam-Bornim e.V. (ATB) Department of Horticultural Engineering Research program Quality and Safety of Food and Feed
08/2011	Luckenwalder Fleischwaren GmbH, quality assurance
03/2011	Luckenwalder Fleischwaren GmbH, quality assurance

Languages

German	Native
English	Fluent
French	Basic skills

School Education

2002-2005	Marie Curie Gymnasium, Ludwigsfelde, Germany (High school)
1998-2002	Goethe-Gymnasium; Trebbin, Germany (High school)
1992-1998	Elementary school

List of Publications

Peer-reviewed publications

1. **Bußler, S., Reinkensmeier, A.; Jeltsch, A., Rawel, H. M., Schlüter, O. (2016):** Plasma processed air as an innovative approach for the synthesis of nitrophenolic compounds *(ready for submittion)*
2. **Bußler, S., Rumpold, B., Jander, E., Rawel, H. M., Schlüter, O. (2016):** Recovery and techno- functionality of flours and proteins from two edible insect species: Mealworm (*Tenebrio molitor*) and black soldier fly (*Hermetia illucens*) larvae. Heliyon, 2 (12): Article e00218.
   Online: http://dx.doi.org/10.1016/j.heliyon.2016.e00218
3. **Bußler, S., Rumpold, B. A., Fröhling, A., Jander, E., Rawel, H. M., Schlüter, O. K. (2016):** Cold atmospheric pressure plasma processing of insect flour from *Tenebrio molitor*: Impact on microbial load and quality attributes in comparison to dry heat treatment. Innovative Food Science and Emerging Technologies, 36: 277-286.
   Online: http://dx.doi.org/10.1016/j.ifset.2016.07.002
4. **Bußler, S., Ehlbeck, J., Schlüter, O. (2016):** Pre-drying treatment of plant related tissues using plasma processed air: Impact on enzyme activity and quality attributes of cut apple and potato. Innovative Food Science and Emerging Technologies. Online: http://dx.org/doi:10.1016/j.ifset.2016.05.007
5. **Reinkensmeier, A.; Steinbrenner, K.; Homann, T.; Bußler, S.; Rohn, S.; Rawel, H. M. (2016):** Monitoring the apple polyphenol oxidase-modulated adduct formation of phenolic and amino compounds. Food Chemistry. 194 (3): 76-85
   Online: http://dx.doi.org/10.1016/j.foodchem.2015.07.145
6. **Bußler, S., Steins, V., Ehlbeck, J., Schlüter, O. (2015):** Impact of thermal treatment versus cold atmospheric plasma processing on the techno-functional protein properties from *Pisum sativum* 'Salamanca', Journal of Food Engineering.
   Online: http://dx.doi.org/10.1016/j.jfoodeng.2015.05.036.
7. **Reinkensmeier, A., Bußler, S., Schlüter, O., Rohn, S., Rawel, H. M. (2015):** Characterization of individual proteins in pea protein isolates and air classified samples. Food Research International.
   Online:http://dx.doi.org/10.1016/j.foodres.2015.05.009.
8. **Bußler, S.; Herppich, W.; Neugart, S.; Schreiner, M.; Ehlbeck, J.; Rohn, S.; Schlüter, O. (2015):** Impact of cold atmospheric pressure plasma on physiology and flavonol glycoside profile of peas (*Pisum sativum* 'Salamanca') Food Research International.
   Online: http://dx.doi.org/10.1016/j.foodres.2015.03.045.
9. **Baier, A. K., Bußler, S., Knorr, D. (2014):** Potential of high isostatic pressure and pulsed electric fields to improve mass transport in pea tissue. Food Research International.
   Online: http://dx.doi.org/10.1016/j.foodres.2014.11.043.
10. **Khanal, B., Knoche, M., Bußler, S., Schlüter, O. (2014):** Evidence for a radial strain gradient in apple fruit cuticles. Planta. (240): 891-897.

Online: http://dx.doi.org/10.1007/s00425-014-2132-0.

Book Chapters

1. **Surowski, B., Bußler, S. & Schlüter, O. (2016).** Cold Plasma Interactions with Liquid and Solid Food Matrices. In N.N. Misra, O. Schlüter & P.J. Cullen (Eds.), Cold Plasma in Food and Agriculture - Fundamentals and Applications (1st ed.): Elsevier, Academic Press.
   Online: http://dx.doi.org/10.1016/B978-0-12-801365-6.00007-X.

Conference Proceedings

1. **Bußler, S.; Schlüter, O. (2015):** Gas-solid interactions of cold atmospheric pressure plasma with complex food matrices. In: Dermesonlouoglou, E.; Giannou, V.; Gogou, E.; Taoukis, P.(eds.): Conference Proceedings Volume I. 29th EFFoST International Conference 2015. (ISBN: 978-618-82196-1-8), p. 464-468.

Presentations at scientific meetings

Invited oral presentations

1. **S. Bußler, Birgit A. Rumpold, A. Fröhling, O. Schlüter (2016).** Improving microbial safety of edible insects and insect-based products by nonthermal atmospheric pressure plasma treatment. 4th International ISEKI_Food Conference, Responsible Research and Innovation in the Food Value Chain, 6 - 8 July 2016, Vienna, Austria.
2. **S. Bußler, O. Schlüter (2015).** Gas-solid interactions of cold atmospheric pressure plasma with complex food matrices, Young researchers-Special Session, 29th EFFoST International Conference, 10-12 November 2015, Athens, Greece.
3. **S. Bußler, O. Schlüter (2015).** Plasma application in food industry: current state and perspectives. WC2015, September 7-10, 2015, Portoroz, Slowenien.
4. **S. Bußler (2014).** Processing of legumes – Impact of various innovative technologies on the properties of pea flour and pea proteins. Cargill R&D Centre Europe, February 18-19, Vilvoorde, Belgium.

Oral presentations

1. **S. Bußler, B. A. Rumpold, A. Fröhling, O. Schlüter (2016).** Improving microbial safety of edible insects and insect-based products by nonthermal atmospheric pressure plasma treatment. 4th International ISEKI_Food Conference, Responsible Research and Innovation in the Food Value Chain, July 6 - 8, Vienna, Austria.
2. **S. Bußler, J. Ehlbeck, O. Schlüter (2015).** Plasma-assisted pre-processing of dried fruits and vegetables, 9th CIGR Section VI International Technical Symposium, 16-20 November 2015, Auckland, New Zealand.
3. **S. Bußler, L. Guérin, B. Rumpold, O. Schlüter (2015).** An approach to optimize protein isolation from alternative sources like meal worms, 9th CIGR Section VI International Technical Symposium, 16-20 November 2015, Auckland, New Zealand.
4. **S. Bußler, H. Rawel, O. Schlüter (2015).** Plasma-protein interactions in solid and liquid model protein matrices, 2015 International Nonthermal Processing Workshop,

12-13 November 2015, Athens, Greece.

5. **S. Bußler, L. Guérin, B. Rumpold, O. Schlüter (2015).** Processing of edible insects: production, fractionation and characterization of flours from meal worm (Tenebrio molitor) and black soldier fly larvae (Hermetia illucens), 29th EFFoST International Conference, 10-12 November 2015, Athens, Greece.
6. **S. Bußler, J. Ehlbeck, O. Schlüter (2015).** Product-specific impact of plasma processed air as a pre-drying procedure for dried fruit and vegetables, 29th EFFoST International Conference, 10-12 November 2015, Athens, Greece.
7. **S. Bußler, B. Rumpold, O. Schlüter (2015).** Gewinnung und Charakterisierung von Mehlfraktionen aus Speiseinsekten. INSECTA 2015, September 21st, Magdeburg, Germany.
8. **S. Bußler, H. Rawel, O. Schlüter (2015).** Plasma-protein interactions: Impact of cold atmospheric pressure plasma on composition, structure and functionality of model proteins. ICEF12, June 14-18, Québec City, Canada.
9. **S. Bußler, A. Weckmüller, H. Rawel, S. Rohn, O. Schlüter (2014).** Flour composition-related control of techno-functional protein properties from *Pisum sativum*. EFFoST Annual Meeting 2014, November 25-28, Uppsala, Sweden.
10. **S. Bußler, J. Ehlbeck, O. Schlüter (2014).** Cold atmospheric pressure plasma for the targeted functionalization of dry bulk materials. V Congreso Internacional de Ciencia y Tecnología de Alimentos 2014, November 17-19, Córdoba, Argentina.
11. **S. Bußler, S. Neugart, M. Schreiner, J. Ehlbeck, S. Rohn, W. B. Herppich, O. Schlüter (2014).** Einsatz von Niedertemperatur-Plasma entlang der Wertschöpfungskette von Leguminosen: Plasma-gestützte Steuerung der Inhaltsstoffprofile und Modifikation der Produkteigenschaften. GDL-Kongress Lebensmitteltechnologie 2014, Octobre 16-18, Rodgau/Frankfurt a. M., Germany.
12. **S. Bußler, U. L. Opara, J. Ehlbeck, O. Schlüter** (2014). Cold atmospheric pressure plasma for the targeted functionalization of dry bulk materials. 18th World Congress of CIGR, International Commission of Agricultural und Biosystems Engineering, September 16-19, Beijing, China.
13. **S. Bußler, J. Ehlbeck, O. Schlüter (2014).** Direct and indirect atmospheric plasma processing for Food preservation and modification - How do product and process interact? ASABE Annual International Meeting, July 13-16, Montreal, Canada.
14. **S. Bußler (2014).** Untersuchungen zu Produkt-Prozess Wechselwirkungen bei der Anwendung kalter Atmosphärendruckplasmen auf Lebensmittel. National ISEKI-Workshop on "hot topics" in the Field of Food Science and Technology, July 1, Berlin, Germany.
15. **S. Bußler, J. Ehlbeck, O. Schlüter (2014).** Functionalization of dry high-protein and high-fibre legume fractions by direct non-thermal atmospheric plasma treatment. Food Structure and Functionality Forum Symposium 2014, March 30-April 2, Amsterdam, The Netherlands.
16. **S. Bußler, A. Heckelmann, D. Knorr, M. Senz, U. Stahl, R. Thomann, H. Rawel, S. Rohn, J. Ehlbeck, O. Schlüter (2014).** Maßgeschneiderte Technologiekonzepte für die Herstellung innovativer Produkte auf Leguminosenbasis: Ausgewählte Beispiele. 49. Vortragstagung DGQ 2014, March 17-18, Kiel, Germany.
17. **S. Bußler, U. Schnabel, J. Ehlbeck, O. Schlüter (2013).** Effects of indirect cold

plasma treatment on enzyme activity and quality parameters of fresh cut and freeze dried apple tissue. 8th International CIGR Technical Symposium 2013, November 3-7, Guangzhou, China.

Poster presentations

1. **S. Bußler, H.M. Rawel, B. Rumpold, A. Fröhling, O. Schlüter (2016).** Impact of Cold Atmospheric Pressure Plasma Treatment on Quality and Safety of Mealworm (*Tenebrio Molitor*) Flour. IFT16, July 16-19, Chicago, USA.
2. **S. Bußler, B. A. Rumpold, A. Fröhling, O. Schlüter (2016).** Improving microbial safety of edible insects and insect-based products by nonthermal atmospheric pressure plasma treatment. 4th International ISEKI_Food Conference, Responsible Research and Innovation in the Food Value Chain, July 6 - 8, Vienna, Austria.
3. **S. Bußler, B. A. Rumpold, A. Fröhling, O. K. Schlüter (2016).** Cold atmospheric pressure plasma treatment as an innovative approach for the decontamination of edible insects and insect-based products. IAFP'S European Symposium on Food Safety, May 11-13, Athens, Greece.
4. **S. Bußler, J. Ehlbeck, O. Schlüter (2015).** Enzyme inactivation by plasma processed air: Impact on quality parameters of fresh cut and freeze dried apple and potato tissue. WC2015, September 7-10, 2015, Portoroz, Slowenien.
5. **S. Bußler, L. Guérin, B. Rumpold, O. Schlüter (2015).** Protein extraction from edible insects: Techno-functional properties of flours and proteins from meal worm and black soldier fly larvae. IFT15, July 11-14, Chicago, USA.
6. **S. Bußler, H. Rawel, J. Ehlbeck and O. Schlüter (2015).** Modification of protein functionalities by the application of cold atmospheric pressure plasma. IFT15, July 11-14, Chicago, USA.
7. **S. Bußler, L. Guérin, B. Rumpold, O. Schlüter (2015).** Insects as a sustainable source of food: Recovery and techno-functionality of meal worm (*Tenebrio molitor*) flour and proteins. ICEF12, June 14-18, Québec City, Canada.
8. **S. Bußler, H. Rawel, J. Ehlbeck, O. Schlüter (2014)**. Cold atmospheric pressure plasma: An innovative tool for the targeted modification of protein functionalities. EFFoST Annual Meeting 2014, November 25-28, Uppsala, Sweden.
9. **S. Bußler, J. Ehlbeck, O. Schlüter (2014).** Impact of conventional thermal versus innovative cold atmospheric plasma processing on the techno-functional protein properties from *Pisum sativum.* 1st Congress on Food Structure Design, October 15-17, Porto, Portugal.
10. **A.Weckmüller, S. Bußler, M. Senz, O. Schlüter, H. M. Rawel, S. Rohn (2014).** Charakterisierung von Proteinen und Protease-Inhibitoren aus *Pisum sativum* - Einflussfaktoren bei der Herstellung innovativer Produkte basierend auf heimischen Leguminosen. 43. Deutscher Lebensmittelchemikertag – GDCh, September 22-24, Gießen, Germany.
11. **S. Bußler, B. Rumpold, A. Fröhling, K. Reineke, O. Schlüter (2014).** Impact of conventional thermal processing and gentle non-thermal plasma treatment on microbial load and protein properties of edible insects. IFT's 2014 Annual Meeting Scientific Program, June 21-24, New Orleans, USA.
12. **J. Jacob, G. Gündüz, S. Bußler, A. Fröhling, C. Theel, O. Schlüter (2014).**

Comparison of the inactivation efficiency of two direct plasma treatments on model food systems. International Food Congress – Novel Approaches in Food Industry - NAFI2014, May 26-29, Kusadasi, Turkey.

13. **S. Bußler, J. Ehlbeck, O. Schlüter (2014).** Non-thermal atmospheric plasma: An innovative approach to modify functional properties of dry bulk materials. 3rd International ISEKI Food Conference 2014, May 21-23, Athens, Greece.
14. **S. Bußler, U. Schnabel, J. Ehlbeck, O. Schlüter (2013).** Impact of direct and indirect cold atmospheric plasma treatment on model food systems. EFFoST Annual Meeting 2013, November 11-15, Bologna, Italy.
15. **S. Bußler, U. Schnabel, J. Ehlbeck, O. Schlüter (2013).** Effects of indirect cold plasma treatment on enzyme activity and quality of fresh cut and freeze dried apple tissue. EFFoST Annual Meeting 2013, November 11-15, Bologna, Italy.
16. **S. Bußler (2013).** Processing of legumes – The influence of high isostatic pressure, pulsed electric fields and high temperature on the properties of pea flour and on yield and structure of soluble pea proteins. EFFoST Annual Meeting 2013, November 11-15, Bologna, Italy.
17. **S. Bußler, U. Schnabel, J. Ehlbeck, O. Schlüter (2013).** Plasma diagnostics: Effects of direct and indirect cold atmospheric plasma treatment on model food systems. 8th International CIGR Technical Symposium 2013, November 3-7, Guangzhou, China.
18. **S. Bußler, U. Schnabel, J. Ehlbeck, O. Schlüter (2013).** Product-process interactions during direct and indirect cold atmospheric plasma treatment as gentle surface sterilization process. iFOOD 2013, October 8-10, Hannover, Germany.
19. **S. Bußler, U. Schnabel, J. Ehlbeck, O. Schlüter (2013).** Impact of indirect cold plasma treatment as gentle food preservation technique on quality and shelf life of fresh cut and freeze dried plant cell tissue. iFOOD 2013, October 8-10, Hannover, Germany.
20. **S. Bußler, A. Heckelmann, D. Knorr (2012).** Influence of high isostatic pressure on the functional properties of peas and pea flour. EFFoST Annual Meeting 2012, November 20-23, Montpellier, France.
21. **S. Bußler, A. Heckelmann, D. Knorr (2011).** Effect of Pulsed Electric Fields on the Yield and Properties of Legume Proteins. EFFoST Annual Meeting 2011, November 9-11, Berlin, Germany.

Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass ich die Dissertation selbständig verfasst habe. Alle benutzten Hilfsmittel und Quellen sind aufgeführt. Weiter erkläre ich, dass ich nicht schon anderweitig einmal die Promotionsabsicht angemeldet oder ein Promotions-eröffnungsverfahren beantragt habe. Veröffentlichungen von irgendwelchen Teilen der vorliegenden Dissertation sind von mir, wie in der vorstehenden Publikationsliste aufgeführt, vorgenommen worden.

Berlin, den 28.07.2016

Sara Bußler