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Influence of Innovative Processing on  $\gamma$ -Aminobutyric Acid (GABA) Contents in Plant Food Materials

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*Published Version:*

Influence of Innovative Processing on  $\gamma$ -Aminobutyric Acid (GABA) Contents in Plant Food Materials / Poojary, Mahesha M.; Dellarosa, Nicolò; Roohinejad, Shahin; Koubaa, Mohamed; Tylewicz, Urszula; Gómez-Galindo, Federico; Saraiva, Jorge A.; Rosa, Marco Dalla; Barba, Francisco J.. - In: COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY. - ISSN 1541-4337. - ELETTRONICO. - 16:5(2017), pp. 895-905. [10.1111/1541-4337.12285]

*Availability:*

This version is available at: <https://hdl.handle.net/11585/627555> since: 2018-02-27

*Published:*

DOI: <http://doi.org/10.1111/1541-4337.12285>

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# Influence of Innovative Processing on $\gamma$ -Aminobutyric Acid (GABA) Contents in Plant Food Materials

Mahesha M. Poojary, Nicolò Dellarosa, Shahin Roohinejad, Mohamed Koubaa, Urszula Tylewicz, Federico Gómez-Galindo, Jorge A. Saraiva, Marco Dalla Rosa, and Francisco J. Barba

**Abstract:** Over the last several decades,  $\gamma$ -aminobutyric acid (GABA) has attracted much attention due to its diverse physiological implications in plants, animals, and microorganisms. GABA naturally occurs in plant materials and its concentrations may vary considerably, from traces up to  $\mu\text{mol/g}$  (dry basis) depending on plant matrix, germination stage, and processing conditions, among other factors. However, due to its important biological activities, considerable interest has been shown by both food and pharmaceutical industries to improve its concentration in plants. Natural and conventional treatments such as mechanical and cold stimulation, anoxia, germination, enzyme treatment, adding exogenous glutamic acid (Glu) or gibberellins, and bacterial fermentation have been shown effective to increase the GABA concentration in several plant materials. However, some of these treatments can modify the nutritional, organoleptic, and/or functional properties of plants. Recent consumer demand for food products which are “healthy,” safe and, having added benefits (nutraceuticals/functional components) has led to explore new ways to improve the content of bioactive compounds while maintaining desirable organoleptic and physicochemical properties. Along this line, nonthermal processing technologies (such as high-pressure processing, pulsed electric fields, and ultrasound, among others) have been shown as means to induce the biosynthesis and accumulation of GABA in plant foods; and the main findings so far reported are presented in this review. Moreover, the most novel tools for the identification of metabolic response in plant materials based on GABA analysis will be also described.

**Keywords:** GABA, high-pressure processing, metabolic response, pulsed electric fields, ultrasound

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## Introduction

$\gamma$ -Aminobutyric acid (GABA), a non-protein amino acid, has attracted significant attention within the past decade, mainly due to its diverse physiological implications in plants, animals, as well as microorganisms (Nikmaram and others 2017). Although there is still conflicting evidence regarding GABA's blood-brain barrier permeability in humans (Boonstra and others 2015), GABA has been regarded as a principal neurotransmitter inhibitor in the mammalian central nervous system, playing a key role in maintaining mental health (Bettler and others 2004). Several clinical and epidemiological studies have suggested that intake of GABA affects a number of physiological processes including acceleration of brain protein synthesis and increasing concentration of plasma growth hormone (Takahara and others 1977; Tujioka and others 2007, 2009; Powers and others 2008; Suwanmanon and Hsieh 2014). In addition, it may also exert several beneficial properties including antihypertensive, antidiabetic, antihypercholesterolemic, anticancer, diuretic, and tranquilizer effects (Adeghate and Ponery 2002; Roohinejad and others 2009a, 2010; Shimada and others 2009; Huang and others 2013; Suwanmanon and Hsieh 2014). Scientific investigations support the hypothesis that it helps in managing stress, pain, and anxiety (Lydiard 2003; Enna and McCarron 2006). It has also shown the potential to reduce obesity

in mice fed a high-fat diet by ameliorating oxidative stress (Xie and others 2014). Owing to its widespread functions, to date, several functional foods/supplements enriched with GABA are available in the market, including web shop giants such as Amazon.com.

In plants, GABA constitutes a considerable portion in the free amino acids pool. GABA is synthesized from 2-oxoglutarate and degraded into succinate by the so-called GABA shunt, bypassing the citric acid cycle enzymes. GABA is accumulated under several biotic and abiotic stress conditions in plants (Sánchez-López and others 2016), although the functional relationship between GABA accumulation and stress remains poorly understood.

As can be seen in Figure 1 (adapted from Bouché and Fromm [2004]), GABA is produced by irreversible decarboxylation of L-Glu, catalyzed by glutamate decarboxylase in response to increased cytosolic  $\text{Ca}^{2+}$  levels when a plant is stressed (Shelp and others 1999). Several scientific studies have shown evidence that GABA plays a key role in multiple physiological processes in plants such as carbon and nitrogen metabolism, cytosolic pH regulation, nitrogen storage, defense against pests, plant microbe interactions,

protection against oxidative stress, osmotic regulation, as well as normal growth and development (Shelp and others 1999; Bouché and Fromm 2004; Gilliham and Tyerman 2016). Detailed information on metabolism and functions of GABA in plants was described in an earlier review (Shelp and others 1999). In recent years, the role of GABA as a signal molecule in plant tissue has also been reported (Bouché and Fromm 2004; Ramesh and others 2015; Gilliham and Tyerman 2016).

GABA naturally occurs in cereals such as brown rice (Sasagawa and others 2006; Roohinejad and others 2009b; Kim and others 2015a; Xia and others 2017), barley (Kihara and others 2007; Imure and others 2009), beans and corn (Oh and others 2003), vegetables such as spinach and potato (Oh and others 2003), chestnuts (Oh and others 2003), fermented foods (Dhawal and others 2012), and dairy products (Siragusa and others 2007). GABA concentrations in the cited products may vary considerably, from traces (2 nmol/g dry basis) to about 700 nmol/g dry basis (Oh and others 2003; Roohinejad and others 2009b). However, owing to its widespread bioactivities, there has been growing interest in improving its concentration in foods.

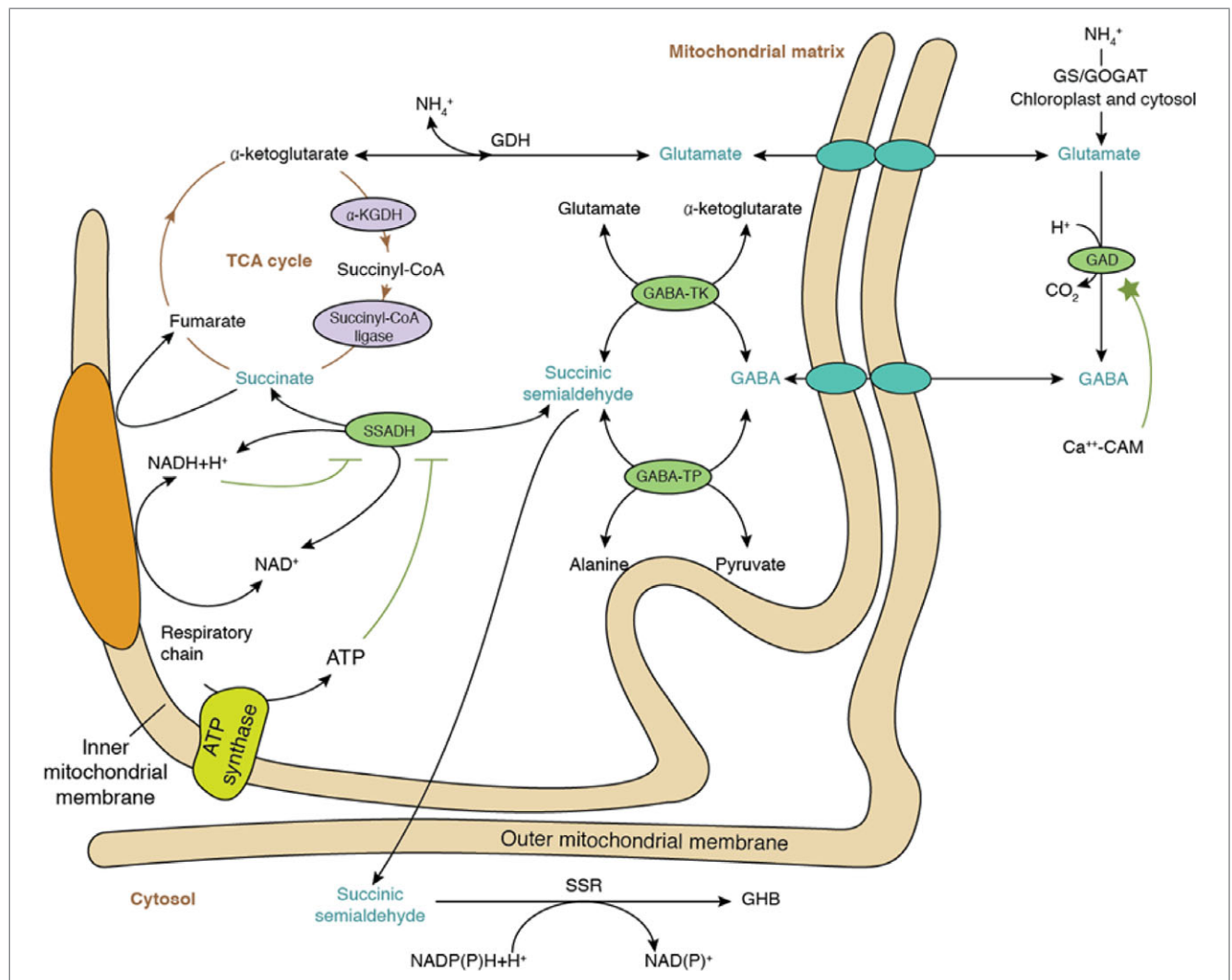


Figure 1—Metabolism and role of GABA. Adapted from (Bouché and Fromm 2004). GS/GOGAT, glutamine-synthetase/glutamate-synthase cycle; GDH, glutamate dehydrogenase; GAD, glutamate decarboxylase (GAD); CaM,  $\text{Ca}^{2+}$ -calmodulin complex; GABA-TK,  $\alpha$ -ketoglutarate-dependent GABA transaminase; GABA-TP, pyruvate-dependent GABA transaminase; SSADH, succinic semialdehyde dehydrogenase; TCA, tricarboxylic acid cycle;  $\alpha$ -KGDH,  $\alpha$ -ketoglutarate dehydrogenase; GHB,  $\gamma$ -hydroxybutyric acid; SSR, semialdehyde reductase.

Naturally, GABA content in plants is increased by a number of biotic (pathogens and parasites) and abiotic stresses (salt, temperature, drought, anoxia, and mechanical force). For example, mechanical and cold stimulation increased GABA concentration in soybeans 20- to 40-fold (Shelp and others 1999) and anoxia increased GABA concentration in rice seedlings by up to 8  $\mu\text{mol/g}$  fresh weight (Reggiani and others 1988). The germination process also increases GABA content considerably in seeds (Martínez-Villaluenga and others 2006; Matsuyama and others 2009; Xu and others 2010; Zhang and others 2014). “Pregermination,” which is described as a soaking or steeping process of cereal in water, is one of the most common techniques applied to increase GABA and other nutrients through endogenous enzyme activation in cereals (Roohinejad and others 2011). Further, several studies have been conducted to improve the GABA content in foods through additional processing such as modifying soaking conditions during germination (Komatsuzaki and others 2007; Das and others 2008; Guo and others 2011; Zhang and others 2014), enzyme treatment (Zhang and others 2006; Das and others 2008), adding exogenous Glu or gibberellins (Zhang and others 2014), and fermentation by lactic acid bacteria (Liao and others 2013; Seo and others 2013).

Nowadays, consumers are demanding food products that are “healthy,” safe, and have added benefits (nutraceuticals/functional components; Cukelj and others 2016). Consequently, industries are looking for innovative processing technologies to produce foods that meet these consumer demands (Bursać-Kovačević and others 2016; Putnik and others 2016a). Certain conventional thermal food processing technologies have shown to reduce the nutritional and bioactive components in foods (Putnik and others 2016b, c). For instance, the high-temperature treatment used for food pasteurization/sterilization may result in a substantial reduction of GABA content (up to 88% to 92%, depending on the matrix and the temperature employed) in foods (Khan and others 2015; Tiansawang and others 2016). For this reason, there is a growing need for utilizing novel non-thermal food processing technologies such as high-pressure processing (HPP), pulsed electric fields (PEF) processing, and ultrasound (US) that can produce high-quality products with better health benefits (natural nutrient retention, improving bioavailability) and desirable organoleptic properties.

Treating a plant tissue at industrial scale is a kind of mechanical/thermal stress to the cell and may therefore affect the nutritional/sensory quality of the final product, depending on the biological reactions *in situ*. Thus, knowledge of how the plant material will be affected during industrial manipulation is of paramount importance for quality assurance and process optimization (Gómez-Galindo and others 2007; Putnik and others 2017a). Recent reports indicate that novel technologies such as HPP, PEF, and US, when applied during postharvest, could act as abiotic elicitors for the biosynthesis and accumulation of plant bioactive molecules. Immediate and late stress responses induced by these technologies were recently reviewed elsewhere (Jacobo-Velázquez and others 2016). The present review focuses on the influence of these technologies on GABA accumulation in plant materials with the aim of developing new functional foods with a high content of these molecules or to be used as potential food additives and/or nutraceuticals. Moreover, the application of novel techniques (such as high-resolution nuclear magnetic resonance, proteomics, and transcriptomics, among others) for the identification of metabolic response in plant materials based on GABA analysis is also discussed in this review.

## Nonthermal Processing

Non-thermal processing refers to those technologies which do not use heat as principal factor in a process, although they might be accompanied by a slight increase in temperature during their application (Misra and others 2017; Putnik and others 2017b). Non-thermal processing technologies such as HPP, PEF, and US have been successfully applied in food preservation due to their ability to inactivate microorganisms and preserving the nutritional and quality attributes (Barba and others 2012, 2015a; Georget and others 2015; Zinoviadou and others 2015). Moreover, the potential of these technologies to control enzymatic complexes (such as activation or inactivation of enzymes) (Eisenmenger and Reyes-De-Corcuera 2009; Chakraborty and others 2014; Terefe and others 2014, 2015a, b) and to extract natural products (Barba and others 2015b; Roselló-Soto, Parniakov, and others 2015; Roohinejad and others 2016; Poojary and others 2017) has also been established. In addition, these technologies have been shown as a promising tool to reduce food contaminants and food allergens and to stimulate microorganisms and metabolic responses (Barba and others 2015a, b). In this review, some of the most relevant findings describing the influence of innovative processing on metabolic response and, particularly, on GABA modifications of plant foods will be described.

### High-pressure processing

HPP, also known as high hydrostatic pressure (HHP), consists on the application of pressures applied in the range of 100 to 1200 MPa, and the pressure increase is generated due to compression of the fluid (Oey and others 2016a; Putnik and others 2017c). An important point for this technology is the versatility that is offered in the process design as well as in food product development due to the possibility of controlling 3 processing parameters (pressure, time, and temperature). In the last 2 decades, HPP has emerged as a potential tool to preserve and improve the quality of food products. As of now, more than 300 industrial high-pressure (HP) models of equipment for food processing are installed around the globe, the main markets being North America, the European Union, Japan, South Korea, Australia, and New Zealand. HP equipment can be also found in China, Peru, and Chile (Barba and others 2016). Traditionally, HPP has been used for food preservation, aiming at producing foods with fewer preservatives and having a longer shelf-life. Being an “emerging” or “novel” technology, the comprehensive applications and prospective utilization of HPP in food science and technology, apart from the well-known food preservation, has not been thoroughly investigated. Nevertheless, now it is well established that HP can induce substantial modifications in cell/tissue, although the mechanistic details of the phenomenon are not well understood. Previous studies have reported that HPP can induce activation or inactivation of inherent enzymes and cause changes in the metabolism and regulation of biochemical and physiological processes in plant tissues (Chakraborty and others 2014; Kim and others 2014; Oey 2016; Figure 2).

Activation and inactivation of certain enzymes may lead to a decrease or an increase in the contents and concentrations of metabolites. Generally, higher pressure levels (>100 MPa) potentially inhibit enzyme activity, while lower levels (<100 MPa) potentially stimulate enzyme activity, by inducing structural/conformational changes or denaturation of substrates (Kim and others 2015b). However, pressure sensitivity of enzymes largely depends on their inherent structure and microenvironmental conditions. As previously mentioned, GABA is accumulated in plant tissues by external stress; since HP can induce stress in plant tissues, this can promote



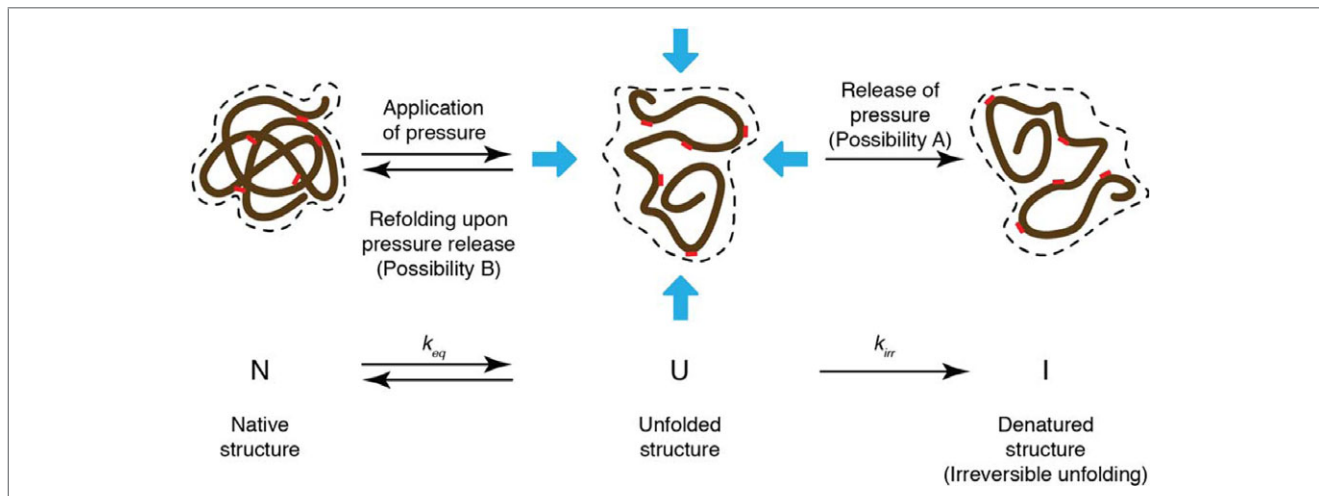


Figure 2—Changes in enzyme structure after high-pressure processing (adapted from Chakraborty and others [2014]). N, native structure; U, unfolded structure; I, irreversible unfolding;  $k_{eq}$ , equilibrium constant;  $k_{irr}$ , irreversible rate constant. Blue arrows indicate high-pressure application.

the GAD activity, consequently enhancing the GABA accumulation in plant tissues. Therefore, the potential of HPP to enhance the accumulation of GABA content in plant foods has been studied (Shigematsu and others 2010a; Ueno and others 2010; Kim and others 2015a, b; Xia and others 2017). Although these reports suggest that HPP influences the activity of GAD, the underlying mechanism has not been well explained.

The HP-induced accumulation of GABA in plant tissues could be due to an increased activity of GAD linked to (i) its structural/conformational changes and/or (ii) changes in its microenvironmental conditions. Apparently, GAD is a stable enzyme, and mild HP treatments may not cause significant changes in its secondary structures. For instance, the crude GAD isolated from HP-treated soybean (200 MPa for 5 min at 25 °C) showed almost similar activity and secondary structure compared with the GAD isolated from the control samples (Ueno 2015). However, the author did not consider the fact of possible reversible unfolding/refolding of native structures after the release of pressure (Chakraborty and others 2014).

Possibly, studying the tertiary structure of enzymes after HPP could reveal additional information on the structure–activity relationship. Yet, the principal reason behind GABA accumulation in HP-treated plant tissues could be due to physical and chemical changes in the cell, induced by HP. It is known that the activity of GAD is stimulated by the  $\text{Ca}^{2+}$ –calmodulin complex. The applied pressure could result in the structural changes of  $\text{Ca}^{2+}$  channel proteins, which, in turn, could facilitate GABA synthesis. Furthermore, it is known that increasing the cytosolic concentration of  $\text{H}^+$  or acidic pH could stimulate GAD activation (Ramputh and Bown 1996; Kinnersley and Turano 2000). HP treatment could enhance the release of  $\text{H}^+$  due to rupture of intracellular organelles or by stimulating certain metabolic processes, which may, in turn, enhance GAD activity (Ueno 2015). Moreover, HPP can also damage cell structures by activating cellulolytic enzymes (Malone and others 2002; Oliveira and others 2012) or by mechanical force, and, as a consequence, it can accelerate the mass transfer of glutamate or GAD and aid-enhanced interaction between Glu and GAD, which could potentially increase the accumulation of GABA. Overall, a detailed investigation on the structural changes in GAD, and the physicochemical changes in the cell, must be investigated to understand the molecular

mechanisms. The hypothetical pathways by which GABA accumulates in plant tissues induced by HPP is shown in Figure 3.

Among many other foods, rice (*Oryza sativa* L.) has been widely investigated, possibly due to its relatively high content of GABA. Brown rice is known as whole grain rice, from which the germ and outer layers have not been removed. When compared to the ordinary milled rice (white rice), brown rice grains are rich in various nutritional compounds, such as dietary fibers, vitamins,  $\gamma$ -oryzanol, and GABA. These functional compounds are found in the germ and bran layers (Roohinejad and others 2009b). GABA contained in brown rice has been reported to be a potential substance to reduce blood cholesterol, although the mechanism of action is not yet clear (Roohinejad and others 2010).

In an early investigation, it has been shown that HP treatment significantly enhanced the accumulation of GABA in brown rice (Kinefuchi and others 1999a). In this study, up to 18.3 mg/100 g DM GABA was extracted from HP-treated (400 MPa) brown rice, while the amount in the control sample was 10.8 mg/100 g DM. However, authors observed a lower level of increase in GABA content at a HP level of 700 MPa (15.9 mg/100 g DM). In a subsequent study, they described the conditions to reduce the viable bacterial counts in the HP-processed rice (Kinefuchi and others 1999b). A soaking time of 10 h at 25 °C and a brown rice-to-water ratio of 1:0.5 (w/w) in the course of pressure treatment was ideal for reducing the bacterial counts (Kinefuchi and others 1999b). In a similar study, an increase of about 3.5-fold in GABA accumulation in HP-treated (200 MPa for 5 min) brown rice (after being kept under saturated humidity at 25 °C for 15 h) was observed compared to control samples (21 mg/100 g against 6 mg/100 g in control samples) (Sasagawa and others 2006). HP treatment at 200 MPa for 10 min was reported to induce accumulation of GABA in brown rice immediately after treatment as well as during subsequent storage (Shigematsu and others 2010a). Similar results were obtained while studying the accumulation of GABA in soybean cotyledons (Ueno and others 2010). These observations clearly suggest that HPP affects the GABA pathway.

The accumulation of GABA in plant tissues could be further improved by the combination of GABA precursor-soaking and HPP. In the case of soybean cotyledon, Glu soaking and HPP enhanced the GABA accumulation considerably (Ueno and others

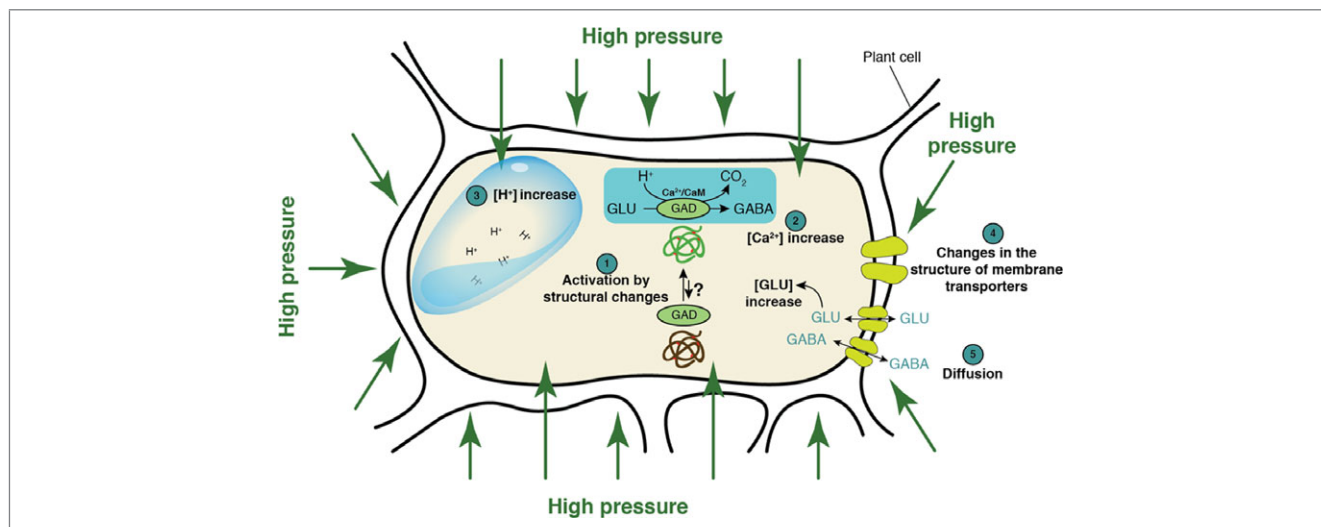


Figure 3–Suggested mechanisms for HP-induced GABA accumulation in plants. (A) High pressure may change the structure and conformation of GAD, thereby stimulating its activity (B) HP may increase Ca<sup>2+</sup>, Glu and (C) H<sup>+</sup> concentration by rupturing internal organelles or activating certain metabolic pathways (D) HP may change the structure of membrane transporters, thereby facilitating import and export of relevant ions and proteins. (E) HP can influence diffusion of enzyme (GAD) and substrates (Glu) enabling a better interaction.

2010). After 2 d of storage, the GABA concentration in Glu-soaked pressurized soybean was 12.35  $\mu\text{mol/g DM}$ , while that in water-soaked sample was 0.90  $\mu\text{mol/g}$  (at 0 d). In another study, HP treatment (200 MPa for 10 min at 25 °C) improved the rate of GABA synthesis in Glu-solution-soaked brown rice during the initial days of preservation (from 0 to 2 d) compared to control samples (Shigematsu and others 2010b). However, this increment was highly dependent on the initial concentration of Glu. During storage, the rate of GABA accumulation increased with increasing concentration of Glu up to 23  $\mu\text{mol/g}$ , while at higher concentrations (23 and 95  $\mu\text{mol/g}$ ), the initial GABA production rate decreased, possibly due to the substrate inhibition effect at higher Glu concentration. This phenomenon was more pronounced in the HP-treated samples compared to control samples, indicating the effect of HP on the activity of GAD. Similarly, a combination of germination and HP treatment was shown to enhance the accumulation of GABA in brown rice (Kim and others 2015a). The authors observed the highest GABA content of 121.21 mg/100 g in the HP-treated (30 MPa for 48 h) 2-d-old germinated seeds.

The HP treatment parameters such as pressure level, temperature, and time may influence the accumulation of GABA in food samples. For instance, a linear increase of GABA concentration of about 21.3 to 106.1 mg/100 g with increasing applied pressure from 0.1 to 100 MPa in nongerminated rough rice was observed (Kim and others 2015b). In the case of germinated rice, interestingly, GABA content (111.4 mg/100 g) increased up to 50 MPa and then decreased afterward to 93.2 mg/100 g. Water absorption during soaking and germination might have affected the activity of GAD and/or the efficacy of the HP treatment. Since the primary effect of HP treatment is the stimulation of GAD activity, a mild treatment is recommended. A pressure >100 MPa may not improve the accumulation of GABA in plant tissues (Xia and others 2017). For instance, the GABA content in germinated brown rice increased from 9.30 mg/100 g to 12.82 mg/100 g after a HP treatment at 100 MPa for 10 min at ambient temperature (18°C) (Xia and others 2017), whereas the increase in pressure level up to 500 MPa did not lead to a significant improvement in GABA accumulation (Xia and others 2017).

## Ultrasound

US treatment is one of the sustainable, green, and nonconventional technologies that has gained increasing popularity in food processing, particularly in tissue homogenization, extraction of bioactive compounds, drying, enzyme inactivation, and filtration (Ramputh and Bown 1996; Koubaa and others 2015, 2016a, b; Roselló-Soto and others 2015; Zhu and others 2017). Several mechanisms have been suggested to be responsible for these operations such as: (1) *Fragmentation* due to interparticle collisions as well as shockwaves produced from the collapsing cavitation bubbles in the liquid (Suslick and Price 1999; Kusters and others 1993, 1994); (2) *Erosion* which is a possible mechanism for improving the extraction through implosion of cavitation bubbles on the surface of leaves that induces erosion of plant structures (Petigny and others 2013; Degrois and others 1974; Suslick and others 1999); (3) *Sonocapillary effect* which refers to the increase of depth and velocity of penetration of liquid into canals and pores under some conditions of sonication (Chemat and others 2017; Mason 2015; Vinatoru 2001); (4) *Sonoporation* for reversible or irreversible cell permeabilization which results in a release of intracellular compounds into the extractive medium (Karshafian and others 2009; Ohta and others 2009; Miller and others 2002; Ugarte-Romero and others 2006; Meullemiestre and others 2016); (5) *Local shear stress* which is generated within the liquid and at the vicinity of solid materials during irradiation of a solid-liquid mixture. Shear forces and turbulences are the result of the oscillation and collapse of cavitation bubbles within the fluid (Vilkhu and others 2011; Veillet and others 2010); and (6) *Detexturation* or destruction of cell structures which helps to improve the extraction of bio-functional materials (Chemat and others 2004; Mason and others 1996). US treatment is determined by the frequency, the power intensity, and the treatment time (Chemat and others 2017).

Limited data are available concerning the effect of ultrasounds on GABA content in the plant tissues. However, a recent report suggests that US could be a potential tool to enhance GABA content in germinating seeds (Yang and others 2015). In this study, soybean seeds were treated by US (0 to 300 W for

30 min at 25 °C) and then allowed to germinate for 5 d. According to the results, the treatment significantly increased germination rate and the GABA content in sprouts. GABA content increased from 83.10 mg/100 g to 119.29 mg/100 g with increasing the US power level, and the seeds pre-treated at 300 W contained up to 43% higher GABA than the control sample. The authors suggested that US might have boosted the GAD activity, thus promoting GABA accumulation in sprouts (Yang and others 2015); nevertheless, the responsible mechanistic aspects were not provided in the study. Since US is a type of stress applicable to plants, it can induce GABA accumulation. However, in this study, the authors noted that US promoted seed germination, which might have indirectly promoted GABA accumulation as germination is accompanied with enhanced GABA synthesis (Komatsuzaki and others 2007; Das and others 2008; Guo and others 2011; Zhang and others 2014). Nevertheless, the study clearly showed that US could be a sustainable tool to produce GABA-enriched foods, and provides a scope for future research.

More recently, the effects of US at different frequencies (0, 30, and 45 kHz) and treatment times (0, 5, 10, 15, 20, 25, and 30 min) on GABA accumulation during several periods of germination (0, 16, and 24 h) of brown rice were studied (Yi and others 2016). The authors found a significant improvement in GABA production of germinated brown rice after US treatments compared to the untreated germinated samples. The authors observed a significant increase in GABA production when treatment time was increased up to 15 min, thus reaching a plateau, and afterwards decreasing the GABA production, although, in any case, GABA production after applying US treatments was significantly higher than the untreated germinated brown rice. Moreover, the authors also found increased production of GABA when US was applied at different frequencies during brown rice germination. They attributed this effect to the ability of US to stimulate the activity of endogenous enzymes, thus speeding up the physiological changes. They found an increased accumulation of GABA when US treatments were applied at 30 kHz compared to 45 kHz. This fact can be explained by the increasingly higher temperatures obtained when high US frequencies are used, thus promoting 1st GABA and, progressively, the decrease of GABA content.

In addition, the impact of US treatments on GABA production in brown rice at different germination times was also studied. The authors found a significant impact of US treatments on GABA content, compared to the untreated samples. They found the maximum GABA accumulation after US treatments at the middle stage of germination (16 h), compared to the later stages of germination (24 h). As the authors stated, during brown rice germination, endogenous enzymes (such as GAD) are activated slowly, thus increasing slowly the production of GABA from glutamate during the time of germination. Therefore, US treatment at different times, can accelerate the rate of endogenous enzyme activation, as well as the decomposition of some biopolymeric compounds, such as starch, and nonstarch polysaccharides, and proteins, thus resulting in the germination of small molecules required for the development of sugars, amino acids, and other nutrients. This fact can explain the maximized GABA production in the middle of the germination after applying US treatments.

## Electrotechnologies

PEF is an emerging technology based on the application of short-time high-voltage pulses to the food product placed between electrodes enclosed in a treatment chamber (Puértolas and others

2016). In order to generate the electric pulses (typically squared or exponentially decaying), a waveform generator at a suitable voltage (generally <80 kV) and current (<1 kA) and a treatment chamber holding the product are needed. PEF process efficacy depends on several factors: with field strength, treatment time, and temperature the most important processing parameters (Oey and others 2016b; Barba and others 2015a).

Over the last few years, PEF has been used for several food applications, particularly important for its effect on microbial inactivation, at high electric field strength, and on improving the mass transfer processes, at lower electric field strength (Toepfl and others 2006; Roohinejad and others 2014a, b; Barba and others 2015b; Mota and others 2017). Both microbial inactivation and improvement of mass transfer phenomena are based on the ability of PEF to induce electroporation phenomena (cell electrical breakdown) in cells of different origins (animal, vegetal, yeast, and so on). Electroporation of the cell membrane depends on the local transmembrane potential, as it affects the mobility of small molecules and ions through the cell membrane, thus damaging it and promoting a stress in the membrane structure, which can promote cell stimulation and/or the production of high-added-value compounds in response to stress (Barba and others 2015a; Mota and others 2017).

Over the last few years, electroporation effect has attracted much interest as it does not require high power consumption, thus increasing the industrial attractiveness of PEF treatment. However, although several studies have been conducted, evaluating microbial inactivation and mass transfer phenomena, the literature about the proper pulse protocol parameters (such as values of electric field strength and pulse duration), required for minimal power consumption, is scarce and further studies are needed (Barba and others 2015a). The electroporation efficiency depends on several pulse parameters (such as pulse duration (10–1000  $\mu$ s), adjustable long pause, type of pulse (monopolar/bipolar), and so on). For instance, pulses of long duration are more effective compared to the shorter ones, especially at room temperature and at moderate electric fields ( $E = 100\text{--}300$  V/cm). Bipolar pulses can cause additional stress in the membrane structure, thus being more effective (Barba and others 2015a).

Several authors have studied the impact of PEF on stimulation of cellular metabolisms with promising results (Nakanishi and others 1998; Carlin 1981; Mattar and others 2015). Recent investigations have shown the potential of PEF to act as elicitors for the biosynthesis and accumulation of plant bioactive molecules (Jacobo-Velázquez and others 2016). However, little is known about the physiological responses of plant tissues subjected to PEF treatment. It has been reported that the metabolic stress response induced by PEF is strongly related to the electric field strength and to the reversibility nature of electroporation phenomena. In fact, the application of PEF at 250 V/cm or higher (100  $\mu$ s pulse width, 60 pulses, 100 Hz) promoted irreversible damages of the membranes leading to a severe loss of the cell viability, while application of 100 V/cm caused only a slight effect on metabolic profiles of fresh-cut apple tissue (Dellarosa and others 2016). The authors showed the metabolites involved in the alteration of the Krebs cycle (such as Glu and GABA) were affected by the application of PEF in different ways. While Glu was increased only by an application of the highest treatment (400 V/cm), this increase could have been the result of a better extraction. The increase of GABA following each PEF treatment with peak values at 250 V/cm could be a result of abiotic stress. In addition, the alteration of the Krebs cycle might also account for the lower heat and CO<sub>2</sub> production.

It was stated that the overall metabolic response of PEF-treated potato tissue was apparently the same as the wounding response regarding the changes in the amino acid pool and the tendency to increase the levels of sterol and galactosyl glycerol-like compounds, while the PEF-specific responses were characterized by changes in the hexose pool that may involve starch and ascorbic acid degradation (Gómez-Galindo and others 2009). Moreover, high levels of quinate and low levels of chlorogenic acid were observed in PEF-treated samples. Concerning Glu, the authors proved that wounding promotes a decrease of its concentration 6 h after the process, while PEF treatment seems to not have any significant effect on this compound (Gómez-Galindo and others 2009).

## Novel Tools for the Identification of Metabolic Response in Plant Materials

The analysis of GABA and other target compounds related to its biological pathway are commonly carried out by liquid chromatography techniques which guarantee high precision and accuracy in terms of separation and quantification of those metabolites (Baum and others 1996). Those targeted methodologies are the ideal approach whenever a food technology is expected to affect defined metabolic pathways. Conversely, when the selection of metabolites is not feasible, an untargeted approach is required (Lopez-Sanchez and others 2015). This typically occurs when an innovative technology provides an external stress input, which might induce an unknown metabolic stress response, leading, for instance, to trigger metabolic pathways that are not ascribable to other similar technologies.

In order to gain insight into the metabolic stress provoked by the application of food process technologies, isothermal microcalorimetric analysis of metabolic heat production has been frequently evaluated in recent years (Wadsö and Gómez Galindo 2009). This unspecific index was demonstrated to be able to grossly describe overall changes induced in plant tissues. It is worth mentioning that, since heat can come from a large number of different sources, the experimental conditions have to be standardized to selectively monitor the effect of the studied technology. The isothermal microcalorimetric assessments, regularly coupled with the analysis of oxygen consumption and carbon dioxide production, were successfully applied to study metabolic consequences by innovative processes such as PEF (Gómez Galindo and others 2008), atmospheric gas-plasma (Tappi and others 2014), and vacuum impregnation (Panarese and others 2014).

To further finely characterize the metabolic effects of a novel technology, a metabolomic untargeted approach can be employed. Metabolomics aims at exploring the holistic metabolic response in plant tissue by simultaneously analyzing tens or hundreds of metabolites and potentially resulting in a comprehensive understanding of the metabolic pathways involved (Cevallos-Cevallos and others 2009). This approach commonly includes the employment of high-throughput techniques, which are able to identify and quantify metabolites belonging to amino acids, organic acids, fatty acids, sugars, alcohols, and other secondary metabolites. Among those techniques, high-resolution nuclear magnetic resonance provides highly reproducible data in wide concentrations ranges and it requires a minimal sample preparation (Laghi and others 2014). On the contrary, high-performance or ultra-performance liquid chromatography (Allwood and Goodacre 2010) or gas chromatography (Lisec and others 2006) along with mass spectroscopy has provided a higher number of observable metabolites at low concentrations.

Although metabolomics assesses the concentrations of metabolites in plant tissue which are directly influenced by endogenous and external inputs (such as food technology), it can be considered downstream of proteomics (Pedreschi and others 2010) and transcriptomics (Valdés and others 2013), which in turn depend on genomics. Proteomics and transcriptomics are fast-evolving techniques, which 1st found applications in the genetic industry for the development of GMOs or new fermentation processes. Besides, to date a limited number of studies have taken into account the relationship between proteins, their transcriptions, and food technologies (Iwahashi and Hosoda 2000; De Angelis and others 2008; Zhao and others 2016). Nevertheless, the combination of metabolomics with proteomic and transcriptomic techniques may lead to a comprehensive understanding of the metabolic alterations promoted by these technologies.

Data output generated by high-throughput methods gives rise to complex matrices, which need reliable robust statistical tools to be investigated. Particularly in the last decade, the development of algorithms and software, especially tailored to explore and find useful information scattered in large datasets, has helped researchers in correlating food technologies to changes in the concentration of metabolites (Trimigno and others 2015). In this context, besides generally accepted unsupervised methods, namely principal component analysis (PCA), the latest adoption of supervised algorithms has allowed to improve the elaboration performance in terms of accuracy and robustness of the found correlations. Different computer-aided tools, mainly based on a projection of latent structures, has been aimed at building models and simultaneously selecting variables. Examples are interval partial least square (iPLS), sparse PLS (sPLS), PLS with genetic algorithm (PLS-GA), PLS with variable importance in projection (PLS-VIP), and interval-extended canonical variable analysis (iECVA) (Mehmood and others 2012; Savorani and others 2013). Those powerful multivariate tools have allowed to overcome problems associated with univariate statistical analysis, namely false positives and false negatives. However, an appropriate validation step using independent datasets is required and fundamental to avoid overfitting and false discoveries (Broadhurst and Kell 2006).

## Conclusions

The available reports clearly suggest that HPP is an excellent tool to enhance accumulation of GABA in plant tissues, thus being a promising strategy to develop better health-beneficial food products. Moreover, the combination of HP and GABA precursor feeding has shown to improve the accumulation of GABA in plant tissues. However, mechanistic evidence is required to confirm the possible influence of HPP in enhancing GABA accumulation in plant tissues. The application of PEF induces stress in the plant tissue, which could be translated to the production of ROS as well as oxygen-consuming pathways. Similar to the results found for HPP, PEF treatment has resulted in a good alternative to enhance the accumulation of GABA in plant tissues (as for apple). However, further studies are required to understand the effect of this technology on the metabolic profile. US treatment at different times and frequencies is an interesting tool to stimulate GABA production. However, GABA production in germinated brown rice is slowed down after a certain US treatment time and frequency. Therefore, there is a need to optimize US processing conditions according to plant material and germination stage.

The overall conclusion from the results found in the available literature is that non-thermal processes (HPP, PEF, and US) can be used to increase the level of GABA in vegetable products, although



at this stage of development, further studies dealing with the optimization of processing conditions, plant material, germination stage, and more are needed. Moreover, the recent developments of innovative, rapid, and reliable analytical methods can boost our knowledge about the metabolic responses induced by novel technologies. The combination of different approaches can lead to a comprehensive understanding of the mechanisms involved in enhancing or inhibiting the accumulation of GABA in plant tissues.

## Acknowledgments

M. M. Poojary is grateful to the Australian Government for being awarded The Australia Awards-Endeavour Research Fellowship under the supervision of Daniel A. Dias, RMIT Univ., Bundoora, Australia. Shahin Roohinejad would like to acknowledge the Alexander von Humboldt Foundation, Germany, for a postdoctoral research fellowship. Jorge A. Saraiva acknowledges FCT/MEC for financial support to the QOPNA Research Unit (FCT UID/QUI/00062/2013), through national funds and, where applicable, cofinanced by the FEDER, within the PT2020 Partnership Agreement.

## Author Contributions

Mahesha M. Poojary researched prior studies, drafted and revised the manuscript. Nicolò Dellarosa researched prior studies and drafted the manuscript. Shahin Roohinejad: revised the manuscript. Mohamed Koubaa prepared the figures and revised the manuscript. Urszula Tylewicz researched prior studies and drafted the manuscript. Federico Gómez-Galindo researched prior studies and drafted the manuscript. Jorge A. Saraiva revised the manuscript. Marco Dalla Rosa researched prior studies and drafted the manuscript. Francisco J. Barba researched prior studies, drafted and revised the manuscript.

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