γ-Aminobutyric acid (GABA) as a potential bioactive food component

Food sources, bioavailability and effects on human cardiometabolic health

Tessa H. De Bie

Propositions

1. A low bioavailability and rapid elimination prevent dietary γ-aminobutyric acid (GABA) from having effects on glucose metabolism (this thesis)

2. The emphasis on the neurological role of GABA has overshadowed the other physiological roles of GABA
(this thesis)

3. Positive health effects of nutrition can rarely be traced back to single food components

4. Dietary supplements and medication require equal quality and safety standards

5. A shift from taxes on income to those on consumption stimulates a more sustainable and healthy lifestyle

6. Focusing on quality instead of yield is the only way for Dutch agriculture to survive

Propositions belonging to the thesis, entitled

γ-Aminobutyric acid (GABA) as a potential bioactive food component: Food sources, bioavailability and effects on human cardiometabolic health

Tessa Helena de Bie
Wageningen, 13 June 2023

γ-Aminobutyric acid (GABA) as a potential bioactive food component

Food sources, bioavailability and effects on human cardiometabolic health

Tessa H. De Bie

Thesis committee

Promotor

Prof.Dr R.F. Witkamp
Professor of Nutritional Biology
Wageningen University & Research

Co-promotors

Dr M.A. Jongsma
Senior researcher, Bioscience
Wageningen University & Research

Dr M.G.J. Balvers
Senior researcher, division of Human Nutrition and Health
Wageningen University & Research

Other members

Prof.Dr Edith Feskens, Wageningen University & Research
Dr Arnaud Bovy, Wageningen University & Research
Dr Mei Wang, Leiden University
Prof.Dr Johan Garssen, Utrecht University

This research was conducted under the auspices of VLAG Graduate School (Biobased, Biomolecular, Chemical, Food, and Nutrition sciences)

γ-Aminobutyric acid (GABA) as a potential bioactive food component

Food sources, bioavailability and effects on human cardiometabolic health

Tessa H. De Bie

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 13 June 2023
at 4 p.m. in the Omnia Auditorium.

Tessa H. De Bie

γ-Aminobutyric acid (GABA) as a potential bioactive food component: Food sources, bioavailability and effects on human cardiometabolic health, 201 pages.

PhD thesis, Wageningen University, Wageningen, the Netherlands (2023)

With references, with summary in English

ISBN 978-94-6447-614-9

DOI https://doi.org/10.18174/589139

Table of contents

Chapter 1

General introduction

Sources for the human omnivorous diet can range from animals to plants and microorganisms. The food products that are derived from those sources contain more than 25.000 components, together called the food metabolome [1]. While intake of some of those components are essential for normal growth, development, or daily functioning, some may confer health benefits beyond that [2]. These bioactive components could offer additional nutritional quality to the human diet. A suboptimal diet has been established as the single greatest risk factor for non-communicable diseases, as it is responsible for 1 in 5 deaths worldwide [3]. To improve the quality of the human diet, more emphasis could be placed on selection or crop fortification that enhances the nutritional quality of fruits and vegetables. Next to improving macronutrient composition, this also concerns the presence of bioactive components with additional health benefits.

However, due to the intensification of agriculture, globalization, population growth, and climate change, biodiversity in food production is decreasing [4]. Crops are traditionally mainly selected based on traits like yield, seed quality, and disease resistance [5]. To provide for our increasing needs, plant breeding and modernization have increased crop yield substantially. However, the global diet is now dependent on only a few of the 300.000 available plant species. In addition, within these species, only a limited number of crop varieties is used. Increasing diversity could potentially improve the quality of human diets [6,7]. The increased awareness among consumers of the benefits of a healthy lifestyle has also fueled the development of functional foods, especially in Japan, northern America and Europe [8]. Lycopene-rich tomatoes and vitamin D-rich mushrooms are examples that can already be found in Dutch supermarkets.

The benefits of adequate intake of essential vitamins and minerals have been well established. Adequate vitamin D status is required for bone health for example [9]. In Europe, health claims regarding vitamin D and improved muscle function and immunity have also been approved by the EFSA [10]. The health effects of supplementation with bioactive 'specialized metabolites' are less well-established [11]. These specialized metabolites are defined as "not essential for survival but enhancing the reproductive success of the host organism under specific environmental conditions" [12]. These specialized metabolites can derive from isoprenyl, phenylpropanoid, nucleotide or amino acid metabolism. In plants, they can have functions related to signaling or defense against biotic or abiotic stresses [13]. Examples are flavonoids, anthocyanins, glucosinolates, indoles and catechins [14]. They are present in fruits and vegetables and are proposed to have antioxidant and anti-inflammatory properties that are beneficial in the prevention or control of non-communicable diseases [11]. This thesis

introduces another specialized metabolite that has the potential to benefit human health: gamma-aminobutyric acid (GABA).

GABA in the diet

GABA is a non-essential amino acid that is not incorporated in proteins. It was first discovered as a nitrogen-containing component of potato tubers in 1949 [15]. When, 7 years later, it was discovered to be an important inhibitory neurotransmitter, the focus of the research field quickly shifted to its function in the human brain [16]. However, GABA also has an important signaling role in plants, where it defends against stress like pests and droughts [17]. Therefore, GABA accumulates in, for example, tomato fruits during the process of fruit development [18]. In the plant, GABA is produced from its precursor glutamic acid by the enzyme glutamic acid decarboxylase (GAD) and it can be broken down again by the enzyme GABA-transaminase to succinic semialdehyde (**figure 1**). GABA is ubiquitously present in plants, but the GABA content can vary substantially [19]. The GABA content has not been determined for all food products, but some interesting examples are already available. Tomatoes have a relatively high GABA content of up to 1.9 g/kg, depending on the variety [20]. Interestingly, tomatoes also contain relatively high amounts of glutamic acid [21]. In tomatoes glutamic acid and GABA are interconnected during the ripening process, increasing and decreasing during the process, respectively [22]. Melons are also in the high range, with GABA concentrations up to 3 g/kg [23]. On the other hand, many other fruits and vegetables contain relatively little GABA [24]. Eggplant for example, a close relative of tomato, contains a 1000x lower GABA concentration than is found in tomato fruits (about 0.0024 g/kg) [25].

The GABA content of food products can be enhanced by fermentation. Microbiota that are used in food fermentation are able to produce GABA from its precursor glutamic acid [26]. Examples of fermented food products with a high GABA content are matured cheeses (up to 10 g/kg) and for example sauerkraut (~0.5 g/kg) [27,28]. In the preparation of sauerkraut, the GABA content increases 30- to 50-fold during fermentation. Fermented soy products like sufu, also contain relatively high amounts of GABA [29]. The ability of the microbiota to produce GABA is also commercially used to specifically enrich food products with GABA, such as GABA-enriched yogurts and teas [30,31].

Together, the intake of these products could lead to a substantial daily GABA intake from the diet. However, data on the average daily GABA intake from food are not available. Next to intake from food products, GABA supplements are also available on the market with many health effects ascribed to it [32]. GABA is especially taken as a supplement to improve sleep quality. Although poorly studied, this is proposed to be related to its function in the brain as an inhibitory neurotransmitter.

GABA in human physiology

In the central nervous system, GABA signaling generally inhibits and hyperpolarizes the cell membrane (**figure 3**) [33]. With this, the GABAergic system plays an important role in for example working memory, sleep/wake regulation, anxiety and executive functions [34–37]. Similar to plants, GABA is produced from its precursor glutamic acid (an excitatory neurotransmitter) by the enzyme glutamic acid decarboxylase (GAD) (**figure 1**) [38]. Two major subclasses of GABA receptors can be distinguished, the $GABA_A$ receptor, a ligand-gated ion channel, and the metabotropic $GABA_B$ receptor which is a G-protein coupled receptor (**figure 3**) [39].

When GABA (or another agonist) binds to the $GABA_A$ receptor, the ion channel is rapidly opened resulting in a flow of chloride ions into the cell that causes hyperpolarization (**figure 3**) [40]. The $GABA_A$ receptor is a pentamer that can potentially consist of any combination of 19 different subunits (α(1–6), β(1–3), γ(1–3), δ, ε, θ, π and ρ(1–3)) [41]. However, in mammals, some combinations of subunits are more abundantly present than others. Most abundant is the combination of two α1, two β2 and one γ2 subunit [42]. The different combinations of subunits give the receptor subtypes specific pharmacological and physiological properties, to suit their function [43].

When GABA (or another agonist) binds to the $GABA_B$ receptor, G-proteins are activated (**figure 3**) [44]. At presynaptic sites, this leads to an inhibition of Ca^{2+} channels to inhibit the release of GABA or other neurotransmitters. At postsynaptic sites, this leads to the opening of K^+ channels and a slow postsynaptic inhibitory current. $GABA_B$ receptors are heterodimers with a B1 and B2 subunit [45]. Only as heterodimer do the subunits form a functional $GABA_B$ receptor [46]. The $GABA_{B1}$ subunit is responsible for the binding of the ligand. As a monomer it is retained in the endoplasmic reticulum and prevented from interacting with the trimeric G-protein complex at the plasma membrane. Only in complex with the $GABA_{B2}$ subunit, the $GABA_{B1}$ subunit is trafficked to the cell surface and available for signaling.

Interestingly, the GABA receptors are also present in peripheral tissues (**figure 2**). Functional $GABA_A$ receptors are present in many tissues, although different combinations of subunits are present as subtypes of the $GABA_A$ receptor depending on its function in that particular tissue [47]. An example of a tissue with a functional $GABA_A$ receptor is the pancreas [48]. The $GABA_A$ subunits that are mainly expressed here are α2, α5, β3 and γ2. As compared to the $GABA_A$ receptor, expression of the $GABA_B$ receptor is organized in a different pattern. $GABA_{B2}$ receptor subunit expression is limited to the adrenal gland, urinary bladder, thyroid, testis and endometrium, while the $GABA_{B1}$ receptor subunit is ubiquitously expressed in peripheral tissues [45,47]. Next to the presence of GABA receptors, GABA is also synthesized in some peripheral tissues [49]. An example is the pancreatic islets, in which GABA is synthesized by GAD [50]. There, GABA reaches similar tissue levels as in the brain, but it is depleted in islets from patients with type 2 and type 1 diabetes [51]. The presence of GABA and

its receptors in the pancreas point towards an important physiological role for GABA in the pancreas.

What is now clear is that in the pancreatic islets, GABA plays a role in regulating hormone release in a paracrine and autocrine manner [52]. Via the $GABA_A$ receptor, GABA can have an inhibitory or excitatory effect on insulin release by depolarization or hyperpolarization of the β-cell membrane [53,54]. This dual effect is possible because of the high intracellular Cl^- concentration that β-cells maintain (unlike mature neurons) [55]. In the case of a high intracellular Cl^- concentration, activation of the $GABA_A$ receptor would lead to an outward flow of chloride ions, which can result in depolarization of the membrane and insulin release [56]. The eventual effect that activation of the $GABA_A$ receptor has depends on the membrane potential, which is influenced by for example the glucose concentration [54]. In this way, GABA can function to maintain equilibrium. An additional inhibitory effect of GABA on insulin release could be mediated by $GABA_B$ receptors. However, it is less clear whether a functional $GABA_B$ receptor is present in the pancreas [57]. While the $GABA_{B1}$ subunit is expressed, the $GABA_{B2}$ subunit is not expressed under basal conditions. Circumstances like the activation of cAMP signaling by, for example, incretin hormones, can lead to the expression of the $GABA_{B2}$ subunit and the formation of a functional $GABA_B$ receptor [58]. Also in the alpha cells, $GABA_A$ receptors are expressed. There, GABA inhibits glucagon release via binding of the $GABA_A$ receptor [59]. In addition, insulin increases the responsiveness of the alpha cells to GABA, by increasing the translocation of $GABA_A$ receptors to the cell membrane [60]. All in all, GABA seems to be a key player in an intricate mechanism to regulate glucose homeostasis.

Potential health effects of GABA

Box diabetes

Diabetes, generally subdivided into type 1 and type 2, is a highly prevalent chronic disease in which the body is unable to regulate blood glucose levels. In 2019, the global prevalence of diabetes was 9.3% of the adult population [61]*. This is expected to increase to 10.9% in 2045. The prevalence increases with age to 19.9% in people aged 65-79 years. Diabetes is diagnosed using cut-off values for fasting plasma glucose (>7 mmol/L) and plasma glucose values 2 hours after an oral glucose tolerance test (>11.1 mmol/L)* [62]*. Individuals with type 1 diabetes can also be screened for islet autoantibodies to prevent symptom development. Type 1 diabetes is an autoimmune disease in which the insulin-producing β-cells are destroyed by immune cells* [63]*. This is accompanied by dysfunctional glucagon-producing α-cells. Due to a lack of insulin production and overproduction of glucagon, glucose homeostasis is dysregulated. This leads to severe complications like nephropathy, cardiovascular disease, dyslipidemia, neuropathy, and foot ulcers* [62]*. Insulin has to be administered to patients to regulate their blood glucose levels.*

The pathogenesis of type 2 diabetes is complex and there are obviously different subtypes, although ultimately causing similar disease symptoms. The current most held view is that in the development of type 2 diabetes, prolonged exposure to dietary glucose and fatty acids in obesity (together with genetic predisposition) leads to inflammation (primarily in the adipose tissue and the liver) and insulin resistance [64]*. Eventually, β-cells fail to produce sufficient insulin to compensate for the insulin resistance that is present. This culminates in chronic hyperglycemia and type 2 diabetes. To control diabetes, lifestyle interventions are recommended that include weight loss and physical activity. Medicinal treatment, preferably also combined with weight management, includes metformin, α-glucosidase inhibitors, sodium–glucose cotransporter 2 inhibitors, glucagon-like peptide 1 receptor agonists, and amylin mimetics* [65]*.*

The gradual transition from adequate glucose homeostasis towards diabetes is usually referred to by the term prediabetes. Although still considered a reversible phase, individuals are at a high risk of developing type 2 diabetes. This phase is characterized by either impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or both [66]*. IFG is associated with hepatic insulin resistance, increased glucagon secretion (hyperglucagonemia), and impaired early-phase insulin secretion, which leads to increased fasting glucose production (gluconeogenesis) and output by the liver* [67]*. IGT is primarily associated with muscle insulin resistance and impaired late-phase insulin secretion, which leads to postprandial hyperglycemia. In both conditions, β-cell dysfunction is already observed, insulin and glucagon secretions are dysfunctional and are no longer able to maintain glucose homeostasis* [68]*. Individuals with prediabetes are recommended to follow a diabetes prevention program* [69]*. This lifestyle and behavioral therapy, including weight loss and physical activity, is shown to be highly effective in reducing the chances of developing diabetes. In a Finnish diabetes prevention program study, the risk reduction was 43% after 7 years* [70]*. However, adherence in general can be an issue and weight loss can be difficult to maintain, in these cases a pharmacological intervention in the form of metformin is recommended in clinical practice, although it is less effective than lifestyle changes* [69]*.*

Results from rodent studies suggest that exogenous administration of GABA can have physiologically relevant effects. Most relevant are the studies that investigated the effects of GABA administration in peripheral tissues since there is no consensus about whether GABA can cross the blood-brain barrier [32]. The effect of GABA has been investigated in rats and mice with experimentally-induced diabetes. For example, models have been used for type 1 diabetes in which streptozotocin is injected before the intervention with GABA [71]. Streptozotocin is specifically toxic to β-cells and leads to a reduction in β-cell mass and function, similar to what is observed in humans with type 1 diabetes. To induce type 2 diabetes, rodents are treated with a high-fat diet before the start of the intervention, with or without a low dose of streptozotocin to also

model the loss in β-cell mass and function that is observed in type 2 diabetes [72]. By administering GABA orally in drinking water or by intraperitoneal injections, the effects of GABA on diabetes symptoms were investigated.

An example of studies in which GABA was investigated in a model for type 1 diabetes, are the studies by Purwana et. al, (2013) in which GABA was given orally, and by Feng et. al, (2017), in which GABA was administered by injection [73,74]. Purwana et. al, (2013) administered GABA for 5 weeks to diabetic mice with transplanted human islets [73]. The administration of GABA led to an increase in β-cell mass and circulating insulin levels, and a decrease in circulating glucagon. This subsequently led to improved blood glucose levels and glucose tolerance. The authors suggested that both activation of the $GABA_A$ and the $GABA_B$ receptor were involved in these effects. Similarly, Feng et. al, (2017) observed that GABA treatment could restore the islet function after streptozotocin treatment by increasing β-cell mass and decreasing the α-cell mass [74].

Similarly, models for type 2 diabetes were used to investigate effects of GABA. Examples are the study by Tian et. al, (2011) which administered GABA orally, and the study by Sohrabipour et. al, (2018) in which GABA was administered by injection [75,76]. Tian et. al, (2011) showed a reduction in fasting blood glucose, improved glucose tolerance and insulin sensitivity in GABA-treated mice [75]. In addition, GABA reduced obesity-induced inflammation, which the authors suggest as a causal mechanism for the observed systemic effects. Sohrabipour et. al, (2018) also showed an improvement in blood glucose, insulin, glucose tolerance and observed changes in gene expression related to a reduction in gluconeogenesis [76].

The available literature extends to other possible peripheral sites of action as well. In spontaneously hypertensive rats, GABA reduced blood pressure after a single dose and after 4 weeks of daily administration [77]. The same authors also show that GABA can reduce blood pressure by reducing noradrenaline release via the activation of $GABA_B$ receptors on sympathetic nerve endings in the vasculature [78].

The effects of exogenous administration of GABA in humans has also been investigated, although less thoroughly and no long-term studies with a robust study design have been published (**table 1**). What has been established is that GABA is absorbed after oral intake and reaches the bloodstream. In 2015, Li et al. were the first to determine the pharmacokinetic profile of oral GABA in humans [79]. A group of 11 volunteers received a single dose of 2 grams of GABA or a placebo. The data showed that GABA is rapidly taken up in the blood, with peak concentrations reached in 0.5 – 1 hour at ~45 times the baseline value and a half-life of 5 hours. In this study, they also showed that the administration of GABA acutely increased insulin and glucagon levels in these participants. This suggests that an increase in GABA plasma level also reflects a physiologic response. Studies with human participants are also available that assess the effects of GABA on blood pressure, with mostly positive results [80–86]. However, these studies show a number of imperfections in their designs. In particular, the

intervention studies used functional foods (GABA-enriched dried Bonito, cheese, milk, bread, rice, and chlorella), often without proper control, for a period of 4 to 12 weeks with a very low dose of 10 to 30 mg GABA.

Aim and outline of this thesis

Research on GABA has so far been largely limited to studies focused on the central nervous system. At the same time, it is clear that GABA may have important peripheral functions and that peripheral concentrations may be influenced by GABA administration. Interestingly, GABA is a component of the human diet and could therefore potentially play a preventive role in metabolic diseases. However, there is still insufficient knowledge to identify GABA as a potentially relevant bioactive food component. Although it has been demonstrated that GABA is taken up in the bloodstream after oral intake, it is unclear how a food matrix affects its bioavailability and whether long-term GABA intake affects the circulating GABA concentration and, ultimately, concentrations at the possible site(s) of action. Next to that, for the determination of the GABA concentration in human plasma, no standard, validated analytical method is available to apply in clinical trials. Finally, while GABA supplements are already marketed with health claims outside the EU, the evidence regarding health effects of oral GABA intake is poor. Therefore, the current thesis aims to investigate the potential of GABA as a dietary plant component with health benefits. In this thesis, we combine research on food sources of GABA with investigating the plasma kinetics and health effects of oral GABA intake.

In **chapter 2** we aimed to identify tomato and potato cultivars and genotypes with a high GABA content. To that end, we screened 72 potato and 91 tomato genotypes for their GABA and glutamic acid content. Next to that, we assessed the effects of domestic cooking methods on the GABA and glutamic acid content of tomato and potato preparations. In **chapter 3** we describe the development and validation of an analytical method using liquid chromatography coupled to tandem mass spectrometry for the simultaneous quantification of GABA and glutamic acid in human plasma. In **chapter 4** we applied this method to determine the influence of a tomato food matrix on the bioavailability and plasma kinetics of oral GABA and glutamic acid in a placebo-controlled, randomized, crossover study with 11 healthy men. In **chapter 5** we explored the health effects of 3 months GABA supplementation in 52 individuals with prediabetes in a parallel, double-blind, randomized, placebo-controlled intervention study. As the primary outcome, the effects of GABA on the postprandial glucose response were investigated. In **chapter 6** the results from chapter 2-5 are discussed.

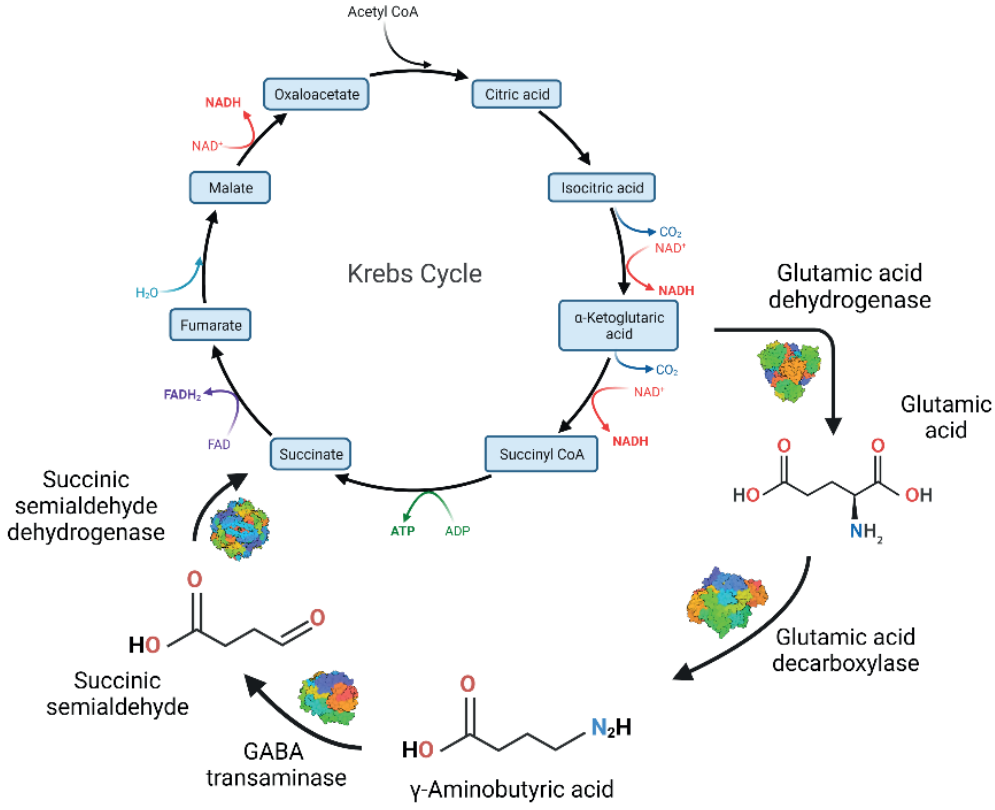


Figure 1: GABA shunt. Showing the synthesis pathway of GABA from glutamic acid.

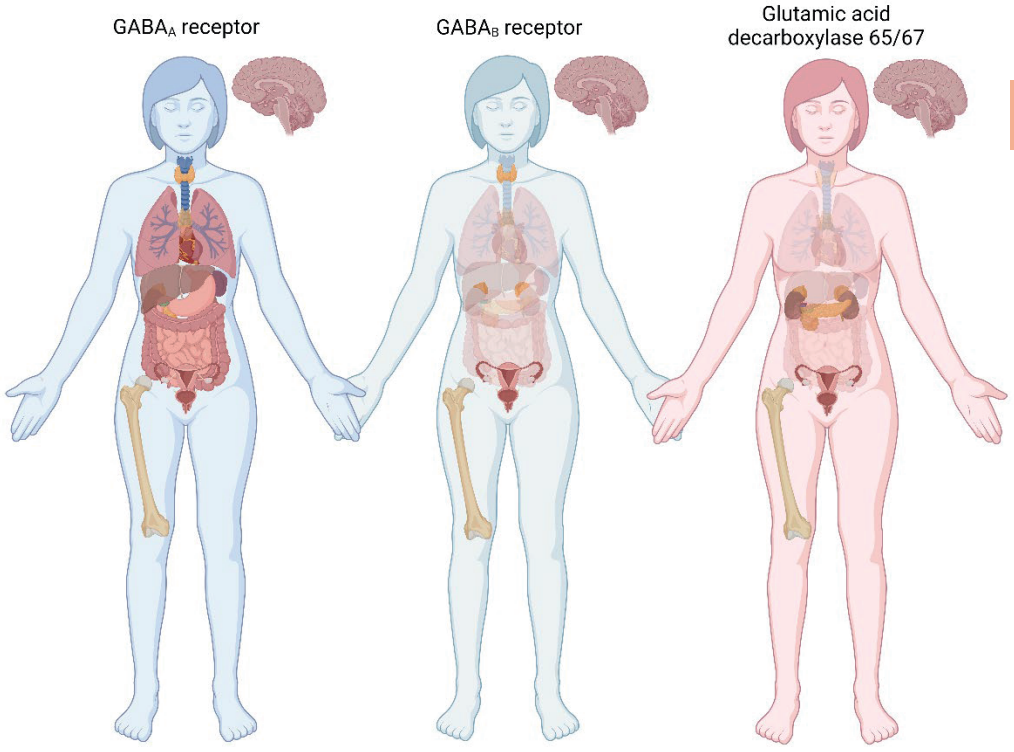


Figure 2: Distribution of GABA receptor and glutamic acid decarboxylase expression throughout the human body. Organs in which expression is present are presented solid.

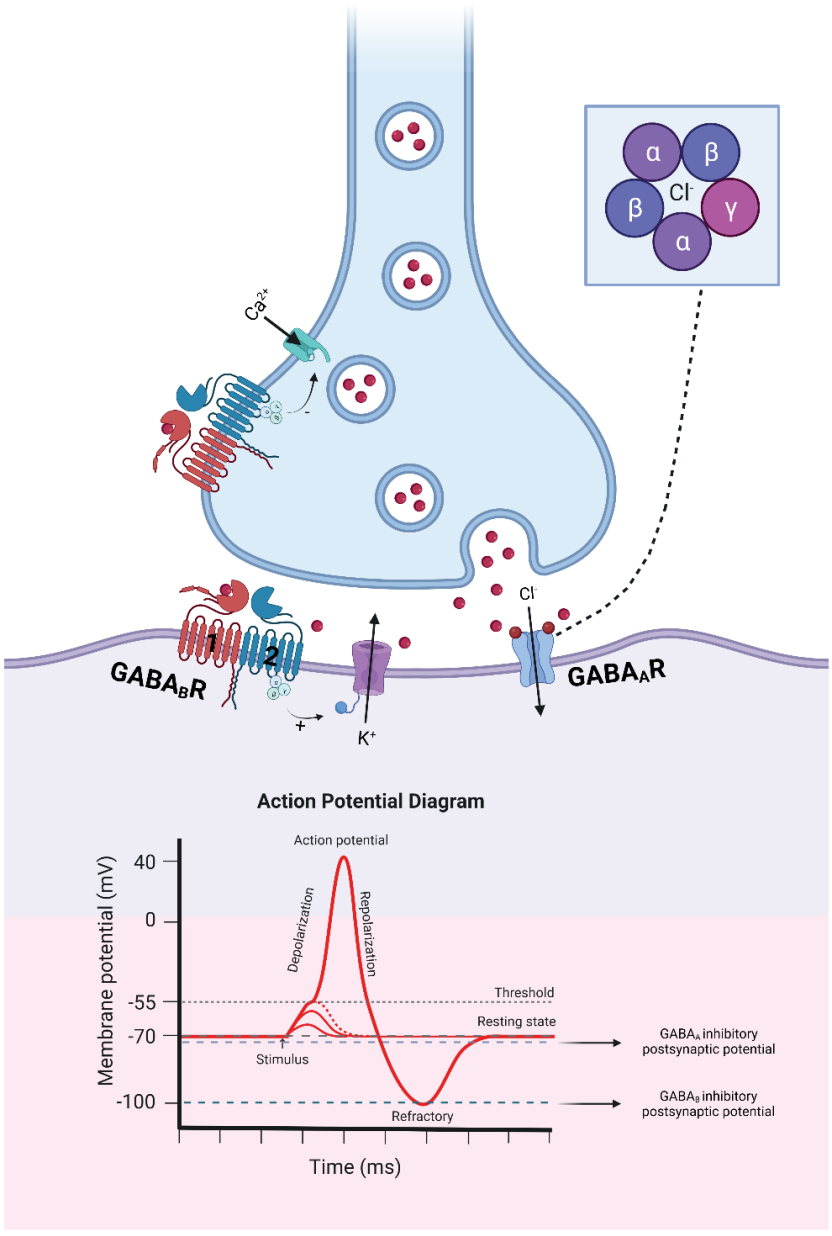


Figure 3: Signal transduction involving GABA at the synapse. Showing a schematic representation the function of ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors with the red dots representing GABA. The action potential diagram shows the depolarization, repolarization and hyperpolarization of the cell membrane after a stimulus. The inhibitory postsynaptic potential of the respective receptors is shown in dotted lines in this diagram. The $GABA_A$ receptor only has a slightly hyperpolarizing effect and could even depolarize depending on the intracellular chloride ion concentration. The $GABA_B$ receptor has far more inhibitory potential.

Table 1: Literature overview of human studies in which oral GABA is administered

Population characteristics : age, health status, sex, number of participants	**Study design and duration**	**Interventional product, dose and control condition**	**Reported effects**[a]	**Reference**
Young (~26) Healthy M/F 11	Three period crossover, washout of 1 week.	1) single dose placebo 2) single dose GABA 3) 7 days repeated dose GABA Single dose: 2g Repeated dose: 2g/3 times a day/7 days	• $AUC_{0\text{-}24h}$: 1451.68 ± 243.12 (±SE) h·ng/ml, single dose • C_{max}: 688 ± 140.7% (±CV%) ng/mL, single dose • T_{max}: 1-1.5h • $T_{1/2}$: 5-5.2h • NSD pharmacokinetics of single and repeated dose • ↑ insulin and glucagon levels • NSD glucose levels • ↓ glycated albumin, repeated dose	Li et al., 2015
~25 Type 1 diabetes M 6	Dose escalation trial Each dose for 3 days	GABA 200 mg 600 mg 1200 mg	• Improved counterregulatory response	Espes et al., 2021
18-52 Healthy 3 M/ 9 F 12	Three period crossover, Washout duration not mentioned. Blood was sampled every 30 minutes for 3 hours. Single dose	1) GABA 5g 2) GABA 10g 3) placebo	• ↑insulin levels • ↑glucagon levels • ↑C-peptide levels	Cavagnini et al., 1982

18-65 Healthy 42 F/3 M 45	Randomised, between-group. Single dose of 5g Repeated dose of 18g for 4 days	1) GABA 2) Water Single dose: 5g Repeated dose: 18g	• ↑growth hormone levels • NSD in prolactin levels	Cavagnini et al., 1980
Young (~24) Healthy M 8	Two period crossover, Washout of 3 days. 30 minutes in resting position at 33 degrees. Single dose	1) 200 mL sports drink 2) 200 mL sports drink with GABA 1g	• ↓delta oesophageal temperature • NSD absolute temperature • NS decrease in sweat loss • ↓heat production • NS increase in heat loss • NSD oxygen uptake	Miyazawa et al., 2012
Young (~23) Healthy M 8	Two-period crossover, Washout of 3 days. 20 min at rest followed by 30 min exercise at 35 degrees.	1) 200 mL sports drink 2) 200 mL sports drink with GABA 1g	• Max GABA plasma levels: 0.8-1.5 nmol/mL (~155 ng/mL) • Basal plasma GABA levels 0.15 nmol/mL (~15 ng/mL) • ↓oesophageal temperature • ↓sweat rate • ↓adrenaline and noradrenaline	Miyazawa et al., 2009
18-24 Healthy 4 M/20 F 24	Double-blind placebo-controlled randomised between-group Single dose	1) GABA dissolved in orange juice 2) Orange juice 800 mg	• No significant difference in attention	Leonte et al., 2018
~37 Poor sleepers (PSQI>6) 7 M/9 F 16 8/group	Single-blind placebo-controlled randomised between-group. 2 test periods per group, washout of 1 week. 1 week	1) GABA 100 mg/d 2) Apocytum venetum leaf extract 50 mg/d 3) GABA 100 mg and Apocytum venetum leaf extract 50 mg 4) Placebo (Dextrin)	• NS decrease in sleep latency • NS increase in non-REM sleep • NS decrease in REM sleep • NSD in awakening • NS increase in delta wave power • NS increase in sleep satisfaction	Yamatsu et al., 2015

Young (~25) Healthy 28 M/ 35 F 63	Single blind, cross-over, randomized, placebo-controlled	1) GABA 2) Dextrin 100 mg/d	• ↓decrease of mood in response to stress • ↓reduced alpha band power after 30 minutes	Yoto et al., 2012
Study 1: 21-35 Healthy 7 M/6 F 13 Study 2: 25-30 acrophobia 5 M/3 F 8	Study 1: Three period crossover, placebo controlled, 1 week washout. Study 2: placebo-controlled randomised between-group. Participants crossed a suspended bridge	Study 1: 1) GABA from lactic acid bacteria 100 mg 2) L-theanine 200 mg 3) Water Study 2: 1) GABA from lactic acid bacteria 100 mg 2) Water	Study 1: • ↑ alpha waves • ↓ beta waves Study 2: • ↑IgA levels	Abdou et al., 2006
20-50 Social drinkers 30 M/30 F 60	Placebo-controlled randomised between-group. Single dose	1) Black sticky rice extract GABA 2) placebo + alcohol 3) GABA + alcohol GABA: 200 mg Alcohol: nale 0.6 g/kg body weight and female 0.4 g/kg body weight	• ↑AUC and peak total stimulation scale score, female • ↓AUC of sedation score, male • ↑AUC of sedation score. Female • ↓blood alcohol concentration, male • NSD blood alcohol concentration, female	Jung et al., 2015

9-13 Autism 5 M/4 F 9	Double-blind, randomized, controlled study 2 weeks	1) GABA Oolong (GABA 279 mg/100 g, L-Theanine 104.48 mg/100 g) 2) Placebo Jiao Gu Lan (GABA 157 mg/100 g, L-Theanine 0 mg/100 g); 3) L-Theanine Gyokuro Green (GABA 156 mg/100 g, L-Theanine 1340 mg/100 g) 4x 300 mL tea/day	• NSD in sensory and motor control	Hannant et al., 2021
~36 Healthy M 24	Double-blind, cross-over, randomized, placebo-controlled Single dose Stress induced with an arithmetic task	1) GABA enriched chocolate (0.28% GABA) 2) Placebo chcolate 10g	• ↓sympathetic activity (LF/HF of HRV) • ↑parasympathetic activity • NS decrease in salivary CgA	Nakamura et al., 2009
~23 Healthy M 7	Double-blind, cross-over, randomized, placebo-controlled Period 1: vegetable tablet Period 2: placebo Single dose	GABA enriched vegetable tablet (31.8mg GABA/tablet) 1 tablet	• NSD in blood pressure, heart rate, stroke volume or cardiac output • ↓LF/HF ratio (sympathetic activity)	Okita et al., 2009

20-65 Mild or moderate hypertension (SBP 130-159 mmHg) F/M? 36	Double blind, placebo controlled, between-group 12 weeks	1) Vinegar and dried Bonito with GABA 2) Vinegar and dried Bonito without GABA 70 mg GABA/d	• ↓in SBP from baseline • ↓in SBP in week 2 and week 14 • ↓in DBP in week, 2, 6, 8, 10, 12, 14 and 16 • ↑chloride from baseline • ↓aspartate aminotransferase, gamma-glutamyl transpeptidases, total protein, lactate dehydrogenase and blood glucose compared to baseline • NSD chloride, aspartate aminotransferase, gamma-glutamyl transpeptidases, total protein, lactate dehydrogenase and blood glucose	Tanaka et al., 2009
40-64 Mild hypertension F/M 46	Randomized, double-blind, placebo-controlled, between-group. 8 weeks	1) High GABA rice (11.2 mg/100g) 2) Low GABA rice (2.7 mg/100g) 150gr/d	• NSD in blood pressure (possible reduction in morning SBP) • NSD in noradrenaline • NSD in liver and renal biomarkers • NSD in glucose and lipid metabolism	Nishimura et al., 2016
20-65 Mild hypertension M 23	Randomized, double-blind, placebo-controlled, between-group. 12 weeks	1) GABA enriched cheese (365 mg/kg) 2) Placebo cheese 50g (16 mg GABA)	• NSD in blood pressure	Pouliot-Mathieu et al., 2013

18-75 Healthy F/M 55	Randomized, crossover, 3-period, controlled feeding study 6 weeks	1) a control, dairy-free diet; 2) a low-fat dairy diet comprising 3 daily servings of 250 mL 1% fat milk; and 3) a regular-fat dairy diet comprising 1 daily serving of 50 g 31% cheddar cheese naturally enriched with GABA (10.1 mg)	• NSD in blood pressure • ↑LDL-cholesterol in diet 3 • ↓HDL-cholesterol in diet 2 • ↑triglycerides in diets 2 and 3 • NSD in CRP	Rancourt-Bouchard et al., 2020
28-81 Mild hypertension 16 F/ 23 M	Randomized, controlled, single-blind trial 12 weeks	1) Fermented skim milk with Lb. casei strain Shirota and Lc. lactis YIT 2027 and sweeteners (10–12 mg/100 ml) 2) Skim milk with L-lactic acid and sweeteners 100 mL per day	• ↓blood pressure after 4 and 12 weeks but not after 8 weeks	Inoue et al., 2003
18-65 Mild hypertension F/M 30	A randomized, controlled, double-blind, crossover trial Three periods of 4 weeks 2 weeks washout	1) Conventional wheat bread 2) low-sodium wheat bread enriched in potassium 3) low-sodium wheat bread rich in potassium, GABA (22.8 mg/100 g), and ACEI peptides 120g/day	• No significant differences in blood pressure	Becerra-Tomas et al., 2015
Mild hypertension F/M 80	Randomized, placebo- controlled, double-blind trial 12 weeks	1) GABA-rich chlorella (20 mg GABA) 2) Placebo	• ↓blood pressure after 8, 10 and 12 weeks	Shimada et al., 2009

a: if multiple intervention products are used, only the effects in the GABA group are stated

References

[1] A. Scalbert, L. Brennan, C. Manach, C. Andres-Lacueva, L.O. Dragsted, J. Draper, S.M. Rappaport, J.J.J. Van Der Hooft, D.S. Wishart, The food metabolome: a window over dietary exposure, The American Journal of Clinical Nutrition. 99 (2014) 1286–1308. https://doi.org/10.3945/AJCN.113.076133.

[2] R.F. Witkamp, Bioactive Components in Traditional Foods Aimed at Health Promotion: A Route to Novel Mechanistic Insights and Lead Molecules?, Annual Review of Food Science and Technology. 13 (2022) 315–336. https://doi.org/10.1146/ANNUREV-FOOD-052720-092845.

[3] A. Afshin, P.J. Sur, K.A. Fay, L. Cornaby, G. Ferrara, J.S. Salama, E.C. Mullany, K.H. Abate, C. Abbafati, Z. Abebe, M. Afarideh, A. Aggarwal, S. Agrawal, T. Akinyemiju, F. Alahdab, U. Bacha, V.F. Bachman, H. Badali, A. Badawi, I.M. Bensenor, E. Bernabe, S.K.K. Biadgilign, S.H. Biryukov, L.E. Cahill, J.J. Carrero, K.M. Cercy, L. Dandona, R. Dandona, A.K. Dang, M.G. Degefa, M. el Sayed Zaki, A. Esteghamati, S. Esteghamati, J. Fanzo, C.S. e. S. Farinha, M.S. Farvid, F. Farzadfar, V.L. Feigin, J.C. Fernandes, L.S. Flor, N.A. Foigt, M.H. Forouzanfar, M. Ganji, J.M. Geleijnse, R.F. Gillum, A.C. Goulart, G. Grosso, I. Guessous, S. Hamidi, G.J. Hankey, S. Harikrishnan, H.Y. Hassen, S.I. Hay, C.L. Hoang, M. Horino, F. Islami, M.D. Jackson, S.L. James, L. Johansson, J.B. Jonas, A. Kasaeian, Y.S. Khader, I.A. Khalil, Y.H. Khang, R.W. Kimokoti, Y. Kokubo, G.A. Kumar, T. Lallukka, A.D. Lopez, S. Lorkowski, P.A. Lotufo, R. Lozano, R. Malekzadeh, W. März, T. Meier, Y.A. Melaku, W. Mendoza, G.B.M. Mensink, R. Micha, T.R. Miller, M. Mirarefin, V. Mohan, A.H. Mokdad, D. Mozaffarian, G. Nagel, M. Naghavi, C.T. Nguyen, M.R. Nixon, K.L. Ong, D.M. Pereira, H. Poustchi, M. Qorbani, R.K. Rai, C. Razo-García, C.D. Rehm, J.A. Rivera, S. Rodríguez-Ramírez, G. Roshandel, G.A. Roth, J. Sanabria, T.G. Sánchez-Pimienta, B. Sartorius, J. Schmidhuber, A.E. Schutte, S.G. Sepanlou, M.J. Shin, R.J.D. Sorensen, M. Springmann, L. Szponar, A.L. Thorne-Lyman, A.G. Thrift, M. Touvier, B.X. Tran, S. Tyrovolas, K.N. Ukwaja, I. Ullah, O.A. Uthman, M. Vaezghasemi, T.J. Vasankari, S.E. Vollset, T. Vos, G.T. Vu, L.G. Vu, E. Weiderpass, A. Werdecker, T. Wijeratne, W.C. Willett, J.H. Wu, G. Xu, N. Yonemoto, C. Yu, C.J.L. Murray, Health effects of dietary risks in 195 countries, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017, The Lancet. 393 (2019) 1958–1972. https://doi.org/10.1016/S0140-6736(19)30041-8.

[4] A.D. Jones, Critical review of the emerging research evidence on agricultural biodiversity, diet diversity, and nutritional status in low- and middle-income countries, Nutrition Reviews. 75 (2017) 769–782. https://doi.org/10.1093/NUTRIT/NUX040.

[5] I. Litrico, C. Violle, Diversity in Plant Breeding: A New Conceptual Framework, Trends Plant Sci. 20 (2015) 604–613. https://doi.org/10.1016/J.TPLANTS.2015.07.007.

[6] K.H.M. Siddique, X. Li, K. Gruber, Rediscovering Asia's forgotten crops to fight chronic and hidden hunger, Nature Plants 2021 7:2. 7 (2021) 116–122. https://doi.org/10.1038/s41477-021-00850-z.

[7] C. Lachat, J.E. Raneri, K.W. Smith, P. Kolsteren, P. Van Damme, K. Verzelen, D. Penafiel, W. Vanhove, G. Kennedy, D. Hunter, F.O. Odhiambo, G. Ntandou-Bouzitou, B. De Baets, D. Ratnasekera, H.T. Ky, R. Remans, C. Termote, Dietary species richness as a measure of food biodiversity and nutritional quality of diets, Proceedings of the National Academy of Sciences of the United States of America. 115 (2018) 127–132. https://doi.org/10.1073/PNAS.1709194115.

[8] C. Karelakis, P. Zevgitis, K. Galanopoulos, K. Mattas, Consumer Trends and Attitudes to Functional Foods, Https://Doi.Org/10.1080/08974438.2019.1599760. 32 (2019) 266–294. https://doi.org/10.1080/08974438.2019.1599760.

[9] M. Emadzadeh, A. Mehdizadeh, P. Sharifan, M. Khoshakhlagh, R. Sahebi, R. Sadeghi, G.A. Ferns, M. Ghayour-Mobarhan, The Effects of Vitamin D Fortified Products on Bone Biomarkers: A Systematic Review and Meta-Analysis, Iranian Journal of Public Health. 51 (2022) 278. https://doi.org/10.18502/IJPH.V51I2.8681.

[10] S. Battault, S.J. Whiting, S.L. Peltier, S. Sadrin, G. Gerber, J.M. Maixent, Vitamin D metabolism, functions and needs: From science to health claims, European Journal of Nutrition. 52 (2013) 429–441. https://doi.org/10.1007/S00394-012-0430-5.

[11] M. da Silveira Vasconcelos, L.M.N. de Oliveira, E.F. Mota, L. de Siqueira Oliveira, N.F. Gomes-Rochette, D.C.S. Nunes-Pinheiro, S.M. Nabavi, D.F. de Melo, Consumption of rich/enrich phytonutrients food and their relationship with health status of population, Phytonutrients in Food: From Traditional to Rational Usage. (2020) 67–101. https://doi.org/10.1016/B978-0-12-815354-3.00006-X.

[12] J.R. Jacobowitz, J.K. Weng, Exploring Uncharted Territories of Plant Specialized Metabolism in the Postgenomic Era, Https://Doi.Org/10.1146/Annurev-Arplant-081519-035634. 71 (2020) 631–658. https://doi.org/10.1146/ANNUREV-ARPLANT-081519-035634.

[13] T.P. Rieseberg, A. Dadras, J.M.R. Fürst-Jansen, A. Dhabalia Ashok, T. Darienko, S. de Vries, I. Irisarri, J. de Vries, Crossroads in the evolution of plant specialized metabolism, Semin Cell Dev Biol. 134 (2023) 37–58. https://doi.org/10.1016/J.SEMCDB.2022.03.004.

[14] C.J. Dillard, J. Bruce German, Phytochemicals: nutraceuticals and human health, J Sci Food Agric. 80 (2000) 1744–1756. https://doi.org/10.1002/1097-0010.

[15] F.C. Steward, γ-Aminobutyric acid : a constituent of the potato tuber?, Science (1979). 110 (1949) 439–440.

[16] A. Bazemore, K.A.C. Elliott, E. Florey, Factor I and γ-Aminobutyric Acid, Nature 1956 178:4541. 178 (1956) 1052–1053. https://doi.org/10.1038/1781052a0.

[17] A.W. Bown, B.J. Shelp, Plant GABA: Not Just a Metabolite, Trends Plant Sci. 21 (2016) 811–813. https://doi.org/10.1016/j.tplants.2016.08.001.

[18] T. Akihiro, S. Koike, R. Tani, T. Tominaga, S. Watanabe, Y. Iijima, K. Aoki, D. Shibata, H. Ashihara, C. Matsukura, K. Akama, T. Fujimura, H. Ezura, Biochemical mechanism on GABA accumulation during fruit development in tomato, Plant and Cell Physiology. 49 (2008) 1378–1389. https://doi.org/10.1093/pcp/pcn113.

[19] N. Bouché, H. Fromm, GABA in plants: Just a metabolite?, Trends Plant Sci. 9 (2004) 110–115. https://doi.org/10.1016/j.tplants.2004.01.006.

[20] T. Saito, C. Matsukura, M. Sugiyama, A. Watahiki, I. Ohshima, Y. Iijima, C. Konishi, T. Fujii, S. Inai, N. Fukuda, S. Nishimura, H. Ezura, Screening for γ-aminobutyric acid (GABA)-rich tomato varieties, Journal of the Japanese Society for Horticultural Science. 77 (2008) 242–250. https://doi.org/10.2503/jjshs1.77.242.

[21] A. Sorrequieta, G. Ferraro, S.B. Boggio, E.M. Valle, Free amino acid production during tomato fruit ripening: a focus on l-glutamate, Amino Acids. 38 (2010) 1523–1532. https://doi.org/10.1007/s00726-009-0373-1.

[22] M. Takayama, H. Ezura, How and why does tomato accumulate a large amount of GABA in the fruit?, Frontiers in Plant Science. 6 (2015) 612. https://doi.org/10.3389/fpls.2015.00612.

[23] T. Toyoizumi, S. Ohba, K. suke Fujii, A. Ikegaya, H. Matsuura, T. Nakajima, Differential GABA concentration gradients are present in the edible parts of greenhouse melon (Cucumis melo L.) during all four seasonal croppings, Biosci Biotechnol Biochem. 83 (2019) 330–338. https://doi.org/10.1080/09168451.2018.1537774.

[24] S.-H. Oh, Y.-J. Moon, C.-H. Oh, γ -Aminobutyric Acid (GABA) Content of Selected Uncooked Foods, Prev Nutr Food Sci. 8 (2003) 75–78. https://doi.org/10.3746/JFN.2003.8.1.075.

[25] H. Horie, A. Ando, T. Saito, The contents of γ-amino butyric acid in eggplant and its accumulation with heat treatment, Nippon Shokuhin Kagaku Kogaku Kaishi. 60 (2013) 661–664. https://doi.org/10.3136/NSKKK.60.661.

[26] J.F. Moore, R. DuVivier, S.D. Johanningsmeier, Formation of γ-aminobutyric acid (GABA) during the natural lactic acid fermentation of cucumber, Journal of Food Composition and Analysis. 96 (2021) 103711. https://doi.org/10.1016/J.JFCA.2020.103711.

[27] B. Redruello, A. Szwengiel, V. Ladero, B. del Rio, M.A. Alvarez, Identification of technological/metabolic/environmental profiles of cheeses with high GABA contents, LWT. (2020) 109603. https://doi.org/10.1016/J.LWT.2020.109603.

[28] X. Yang, W. Hu, Z. Xiu, A. Jiang, X. Yang, G. Saren, Y. Ji, Y. Guan, K. Feng, Microbial Community Dynamics and Metabolome Changes During Spontaneous Fermentation

of Northeast Sauerkraut From Different Households, Front Microbiol. 11 (2020) 1878. https://doi.org/10.3389/FMICB.2020.01878/BIBTEX.

[29] L. Xu, W.X. Cai, B.J. Xu, A Systematic Assesment on Vitamins (B2, B12) and GABA Profiles in Fermented Soy Products Marketed in China, Journal of Food Processing and Preservation. 41 (2017) e13126. https://doi.org/10.1111/JFPP.13126.

[30] T. Hinton, G.A.R. Johnston, GABA-enriched teas as neuro-nutraceuticals, Neurochem Int. 141 (2020) 104895. https://doi.org/10.1016/J.NEUINT.2020.104895.

[31] F.S. Hussin, S.Y. Chay, A.S.M. Hussin, W.Z. Wan Ibadullah, B.J. Muhialdin, M.S. Abd Ghani, N. Saari, GABA enhancement by simple carbohydrates in yoghurt fermented using novel, self-cloned Lactobacillus plantarum Taj-Apis362 and metabolomics profiling, Scientific Reports 2021 11:1. 11 (2021) 1–12. https://doi.org/10.1038/s41598-021-88436-9.

[32] E. Boonstra, R. de Kleijn, L.S. Colzato, A. Alkemade, B.U. Forstmann, S. Nieuwenhuis, Neurotransmitters as food supplements: the effects of GABA on brain and behavior, Front Psychol. 6 (2015) 1520. https://doi.org/10.3389/fpsyg.2015.01520.

[33] W. Kilb, Development of the GABAergic system from birth to adolescence, Neuroscientist. 18 (2012) 613–630. https://doi.org/10.1177/1073858411422114.

[34] C. Constantinidis, G. V. Williams, P.S. Goldman-Rakic, A role for inhibition in shaping the temporal flow of information in prefrontal cortex, Nature Neuroscience. 5 (2002) 175–180. https://doi.org/10.1038/NN799.

[35] C. Gottesmann, GABA mechanisms and sleep, Neuroscience. 111 (2002) 231–239. https://doi.org/10.1016/S0306-4522(02)00034-9.

[36] T. Oishi, A. Mikami, K. Kubota, Local injection of bicuculline into area 8 and area 6 of the rhesus monkey induces deficits in performance of a visual discrimination GO/NO-GO task, Neuroscience Research. 22 (1995) 163–177. https://doi.org/10.1016/0168-0102(96)80436-6.

[37] A. V. Kalueff, D.J. Nutt, Role of GABA in anxiety and depression, Depression and Anxiety. 24 (2007) 495–517. https://doi.org/10.1002/DA.20262.

[38] S.E. Lee, Y. Lee, G.H. Lee, The regulation of glutamic acid decarboxylases in GABA neurotransmission in the brain, Archives of Pharmacal Research 2019 42:12. 42 (2019) 1031–1039. https://doi.org/10.1007/S12272-019-01196-Z.

[39] M. Watanabe, K. Maemura, K. Kanbara, T. Tamayama, H. Hayasaki, GABA and GABA Receptors in the Central Nervous System and Other Organs, Int Rev Cytol. 213 (2002) 1–47. https://doi.org/10.1016/S0074-7696(02)13011-7.

[40] M.K. Akinci, P.R. Schofield, Widespread expression of GABAA receptor subunits in peripheral tissues, Neurosci Res. 35 (1999) 145–153. https://doi.org/10.1016/S0168-0102(99)00078-4.

[41] E. Sigel, M.E. Steinmann, Structure, function, and modulation of GABA(A) receptors, The Journal of Biological Chemistry. 287 (2012) 40224–40231. https://doi.org/10.1074/JBC.R112.386664.

[42] R.W. Olsen, W. Sieghart, International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update, Pharmacological Reviews. 60 (2008) 243–260. https://doi.org/10.1124/PR.108.00505.

[43] A. Garifulina, T. Friesacher, M. Stadler, E.M. Zangerl-Plessl, M. Ernst, A. Stary-Weinzinger, A. Willam, S. Hering, β subunits of GABAA receptors form proton-gated chloride channels: Insights into the molecular basis, Communications Biology 2022 5:1. 5 (2022) 1–10. https://doi.org/10.1038/s42003-022-03720-2.

[44] B. Bettler, K. Kaupmann, J. Mosbacher, M. Gassmann, Molecular structure and physiological functions of GABAB receptors, Physiological Reviews. 84 (2004) 835–867. https://doi.org/10.1152/PHYSREV.00036.2003.

[45] A.R. Calver, A.D. Medhurst, M.J. Robbins, K.J. Charles, M.L. Evans, D.C. Harrison, M. Stammers, S.A. Hughes, G. Hervieu, A. Couve, S.J. Moss, D.N. Middlemiss, M.N. Pangalos, The expression of GABAB1 and GABAB2 receptor subunits in the cNS differs from that in peripheral tissues, Neuroscience. 100 (2000) 155–170. https://doi.org/10.1016/S0306-4522(00)00262-1.

[46] T. Galvez, B. Duthey, J. Kniazeff, J. Blahos, G. Rovelli, B. Bettler, L. Prézeau, J.P. Pin, Allosteric interactions between GB1 and GB2 subunits are required for optimal GABAB receptor function, The EMBO Journal. 20 (2001) 2152–2159. https://doi.org/10.1093/EMBOJ/20.9.2152.

[47] L. Fagerberg, B.M. Hallstrom, P. Oksvold, C. Kampf, D. Djureinovic, J. Odeberg, M. Habuka, S. Tahmasebpoor, A. Danielsson, K. Edlund, A. Asplund, E. Sjostedt, E. Lundberg, C.A.K. Szigyarto, M. Skogs, J. Ottosson Takanen, H. Berling, H. Tegel, J. Mulder, P. Nilsson, J.M. Schwenk, C. Lindskog, F. Danielsson, A. Mardinoglu, A. Sivertsson, K. Von Feilitzen, M. Forsberg, M. Zwahlen, I. Olsson, S. Navani, M. Huss, J. Nielsen, F. Ponten, M. Uhlen, Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics, Molecular & Cellular Proteomics : MCP. 13 (2014) 397–406. https://doi.org/10.1074/MCP.M113.035600.

[48] S. v Korol, Z. Jin, Y. Jin, A.K. Bhandage, A. Tengholm, N.R. Gandasi, S. Barg, D. Espes, P.-O. Carlsson, D. Laver, B. Birnir, Functional Characterization of Native, High-Affinity GABAAReceptors in Human Pancreatic β Cells., EBioMedicine. 0 (2018). https://doi.org/10.1016/j.ebiom.2018.03.014.

[49] B. Zhang, A. Vogelzang, M. Miyajima, Y. Sugiura, Y. Wu, K. Chamoto, R. Nakano, R. Hatae, R.J. Menzies, K. Sonomura, N. Hojo, T. Ogawa, W. Kobayashi, Y. Tsutsui, S. Yamamoto, M. Maruya, S. Narushima, K. Suzuki, H. Sugiya, K. Murakami, M. Hashimoto, H. Ueno, T. Kobayashi, K. Ito, T. Hirano, K. Shiroguchi, F. Matsuda, M. Suematsu, T. Honjo, S. Fagarasan, B cell-derived GABA elicits IL-10+ macrophages to limit anti-tumour immunity, Nature 2021 599:7885. 599 (2021) 471–476. https://doi.org/10.1038/s41586-021-04082-1.

[50] Y. Okada, H. Taniguchi, C. Shimada, High Concentration of GABA and High Glutamate Decarboxylase Activity in Rat Pancreatic Islets and Human Insulinoma, Science. 194 (1976) 620–622. https://doi.org/10.1126/SCIENCE.185693.

[51] D. Menegaz, D.W. Hagan, J. Almaça, C. Cianciaruso, R. Rodriguez-Diaz, J. Molina, R.M. Dolan, M.W. Becker, P.C. Schwalie, R. Nano, F. Lebreton, C. Kang, R. Sah, H.Y. Gaisano, P.-O. Berggren, S. Baekkeskov, A. Caicedo, E.A. Phelps, Mechanism and effects of pulsatile GABA secretion from cytosolic pools in the human beta cell, Nat Metab. 1 (2019) 1110–1126. https://doi.org/10.1038/s42255-019-0135-7.

[52] D.W. Hagan, S.M. Ferreira, G.J. Santos, E.A. Phelps, The role of GABA in islet function, Front Endocrinol (Lausanne). 13 (2022). https://doi.org/10.3389/FENDO.2022.972115.

[53] M. Braun, R. Ramracheya, M. Bengtsson, A. Clark, J.N. Walker, P.R. Johnson, P. Rorsman, γ-Aminobutyric Acid (GABA) Is an Autocrine Excitatory Transmitter in Human Pancreatic β-Cells, Diabetes. 59 (2010) 1694–1701. https://doi.org/10.2337/DB09-0797.

[54] H. Dong, M. Kumar, Y. Zhang, A. Gyulkhandanyan, Y.Y. Xiang, B. Ye, J. Perrella, A. Hyder, N. Zhang, M. Wheeler, W.Y. Lu, Q. Wang, Gamma-aminobutyric acid up- and downregulates insulin secretion from beta cells in concert with changes in glucose concentration, Diabetologia. 49 (2006) 697–705. https://doi.org/10.1007/S00125-005-0123-1.

[55] M. Eberhardson, S. Patterson, E. Grapengiesser, Microfluorometric analysis of Cl− permeability and its relation to oscillatory Ca2+ signalling in glucose-stimulated pancreatic β-cells, Cell Signal. 12 (2000) 781–786. https://doi.org/10.1016/S0898-6568(00)00122-4.

[56] M. Di Fulvio, L. Aguilar-Bryan, Chloride transporters and channels in β-cell physiology: revisiting a 40-year-old model, Biochemical Society Transactions. 47 (2019) 1843–1855. https://doi.org/10.1042/BST20190513.

[57] N.L. Brice, A. Varadi, S.J.H. Ashcroft, E. Molnar, Metabotropic glutamate and GABAB receptors contribute to the modulation of glucose-stimulated insulin secretion in pancreatic beta cells, Diabetologia 2002 45:2. 45 (2014) 242–252. https://doi.org/10.1007/S00125-001-0750-0.

[58] L. Rachdi, A. Maugein, S. Pechberty, M. Armanet, J. Hamroune, P. Ravassard, S. Marullo, O. Albagli, R. Scharfmann, Regulated expression and function of the GABAB

receptor in human pancreatic beta cell line and islets, Scientific Reports 2020 10:1. 10 (2020) 1–10. https://doi.org/10.1038/s41598-020-69758-6.

[59] P. Rorsman, P.O. Berggren, K. Bokvist, H. Ericson, H. Möhler, C.G. Östenson, P.A. Smith, Glucose-inhibition of glucagon secretion involves activation of GABAA-receptor chloride channels, Nature 1989 341:6239. 341 (1989) 233–236. https://doi.org/10.1038/341233a0.

[60] E. Xu, M. Kumar, Y. Zhang, W. Ju, T. Obata, N. Zhang, S. Liu, A. Wendt, S. Deng, Y. Ebina, M.B. Wheeler, M. Braun, Q. Wang, Intra-islet insulin suppresses glucagon release via GABA-GABAA receptor system, Cell Metab. 3 (2006) 47–58. https://doi.org/10.1016/J.CMET.2005.11.015.

[61] P. Saeedi, I. Petersohn, P. Salpea, B. Malanda, S. Karuranga, N. Unwin, S. Colagiuri, L. Guariguata, A.A. Motala, K. Ogurtsova, J.E. Shaw, D. Bright, R. Williams, Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition, Diabetes Res Clin Pract. 157 (2019) 107843. https://doi.org/10.1016/J.DIABRES.2019.107843.

[62] A.D. Association, Standards of Medical Care in Diabetes—2010, Diabetes Care. 33 (2010) S11–S61. https://doi.org/10.2337/DC10-S011.

[63] J. Ilonen, J. Lempainen, R. Veijola, The heterogeneous pathogenesis of type 1 diabetes mellitus, (n.d.). https://doi.org/10.1038/s41574-019-0254-y.

[64] R.A. DeFronzo, E. Ferrannini, L. Groop, R.R. Henry, W.H. Herman, J.J. Holst, F.B. Hu, C.R. Kahn, I. Raz, G.I. Shulman, D.C. Simonson, M.A. Testa, R. Weiss, Type 2 diabetes mellitus, Nat Rev Dis Primers. (2015) 15019. https://doi.org/10.1038/nrdp.2015.19.

[65] A.D.A.P.P. Committee, 8. Obesity and Weight Management for the Prevention and Treatment of Type 2 Diabetes: Standards of Medical Care in Diabetes—2022, Diabetes Care. 45 (2022) S113–S124. https://doi.org/10.2337/DC22-S008.

[66] G. Bock, C.D. Man, M. Campioni, E. Chittilapilly, R. Basu, G. Toffolo, C. Cobelli, R. Rizza, Pathogenesis of Pre-Diabetes, Diabetes. 55 (2006) 3536–3549. https://doi.org/10.2337/DB06-0319.

[67] A. Święcicka-Klama, K. Połtyn-Zaradna, A. Szuba, K. Zatońska, The Natural Course of Impaired Fasting Glucose, Adv Exp Med Biol. 1324 (2021) 41–50. https://doi.org/10.1007/5584_2020_571.

[68] M. Abdul-Ghani, D. Tripathy, R. DeFronzo, Contributions of beta-cell dysfunction and insulin resistance to the pathogenesis of impaired glucose tolerance and impaired fasting glucose, Diabetes Care. 29 (2006) 1130–1139. https://doi.org/10.2337/DIACARE.2951130.

[69] A.D.A.P.P. Committee, 3. Prevention or Delay of Type 2 Diabetes and Associated Comorbidities: Standards of Medical Care in Diabetes—2022, Diabetes Care. 45 (2022) S39–S45. https://doi.org/10.2337/DC22-S003.

[70] J. Lindström, P. Ilanne-Parikka, M. Peltonen, S. Aunola, J.G. Eriksson, K. Hemiö, H. Hämäläinen, P. Härkönen, S. Keinänen-Kiukaanniemi, M. Laakso, A. Louheranta, M. Mannelin, M. Paturi, J. Sundvall, T.T. Valle, M. Uusitupa, J. Tuomilehto, Sustained reduction in the incidence of type 2 diabetes by lifestyle intervention: follow-up of the Finnish Diabetes Prevention Study, The Lancet. 368 (2006) 1673–1679. https://doi.org/10.1016/S0140-6736(06)69701-8.

[71] J.A. Pearson, F.S. Wong, L. Wen, The importance of the Non Obese Diabetic (NOD) mouse model in autoimmune diabetes, Journal of Autoimmunity. 66 (2016) 76–88. https://doi.org/10.1016/J.JAUT.2015.08.019.

[72] A. Heydemann, An Overview of Murine High Fat Diet as a Model for Type 2 Diabetes Mellitus, Journal of Diabetes Research. 2016 (2016). https://doi.org/10.1155/2016/2902351.

[73] I. Purwana, J. Zheng, X. Li, M. Deurloo, D.O. Son, Z. Zhang, C. Liang, E. Shen, A. Tadkase, Z.-P. Feng, Y. Li, C. Hasilo, S. Paraskevas, R. Bortell, D.L. Greiner, M. Atkinson, G.J. Prud'homme, Q. Wang, GABA promotes human β-cell proliferation and modulates glucose homeostasis, Diabetes. 63 (2014) 4197–4205. https://doi.org/10.2337/db14-0153.

[74] A.L. Feng, Y.-Y. Xiang, L. Gui, G. Kaltsidis, Q. Feng, W.-Y. Lu, Paracrine GABA and insulin regulate pancreatic alpha cell proliferation in a mouse model of type 1 diabetes, Diabetologia. 60 (2017) 1033–1042. https://doi.org/10.1007/s00125-017-4239-x.

[75] J. Tian, H.N. Dang, J. Yong, W.-S. Chui, M.P.G. Dizon, C.K.Y. Yaw, D.L. Kaufman, Oral Treatment with γ-Aminobutyric Acid Improves Glucose Tolerance and Insulin Sensitivity by Inhibiting Inflammation in High Fat Diet-Fed Mice, PLoS One. 6 (2011) e25338. https://doi.org/10.1371/journal.pone.0025338.

[76] S. Sohrabipour, M.R. Sharifi, A. Talebi, M. Sharifi, N. Soltani, GABA dramatically improves glucose tolerance in streptozotocin-induced diabetic rats fed with high-fat diet, Eur J Pharmacol. 826 (2018) 75–84. https://doi.org/10.1016/J.EJPHAR.2018.01.047.

[77] K. Hayakawa, M. Kimura, K. Kasaha, K. Matsumoto, H. Sansawa, Y. Yamori, Effect of a γ-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar–Kyoto rats, British Journal of Nutrition. 92 (2004) 411. https://doi.org/10.1079/BJN20041221.

[78] K. Hayakawa, M. Kimura, K. Kamata, Mechanism underlying γ-aminobutyric acid-induced antihypertensive effect in spontaneously hypertensive rats, Eur J Pharmacol. 438 (2002) 107–113. https://doi.org/10.1016/S0014-2999(02)01294-3.

[79] J. Li, Z. Zhang, X. Liu, Y. Wang, F. Mao, J. Mao, X. Lu, D. Jiang, Y. Wan, J.-Y. Lv, G. Cao, J. Zhang, N. Zhao, M. Atkinson, D.L. Greiner, G.J. Prud'homme, Z. Jiao, Y. Li, Q. Wang, Study of GABA in Healthy Volunteers: Pharmacokinetics and Pharmacodynamics, Front Pharmacol. 6 (2015) 260. https://doi.org/10.3389/fphar.2015.00260.

[80] H. Tanaka, K. Watanabe, M. Ma, M. Hirayama, T. Kobayashi, H. Oyama, Y. Sakaguchi, M. Kanda, M. Kodama, Y. Aizawa, The Effects of gamma-Aminobutyric Acid, Vinegar, and Dried Bonito on Blood Pressure in Normotensive and Mildly or Moderately Hypertensive Volunteers., J Clin Biochem Nutr. 45 (2009) 93–100. https://doi.org/10.3164/jcbn.09-04.

[81] M. Nishimura, S. Yoshida, M. Haramoto, H. Mizuno, T. Fukuda, H. Kagami-Katsuyama, A. Tanaka, T. Ohkawara, Y. Sato, J. Nishihira, Effects of white rice containing enriched gamma-aminobutyric acid on blood pressure, J Tradit Complement Med. 6 (2016) 66–71. https://doi.org/10.1016/J.JTCME.2014.11.022.

[82] K. Pouliot-Mathieu, C. Gardner-Fortier, S. Lemieux, D. St-Gelais, C.P. Champagne, J.-C. Vuillemard, Effect of cheese containing gamma-aminobutyric acid-producing lactic acid bacteria on blood pressure in men, PharmaNutrition. 1 (2013) 141–148. https://doi.org/10.1016/J.PHANU.2013.06.003.

[83] M. Rancourt-Bouchard, I. Gigleux, V. Guay, A. Charest, D. Saint-Gelais, J.C. Vuillemard, B. Lamarche, P. Couture, Effects of regular-fat and low-fat dairy consumption on daytime ambulatory blood pressure and other cardiometabolic risk factors: a randomized controlled feeding trial, Am J Clin Nutr. 111 (2020) 42–51. https://doi.org/10.1093/AJCN/NQZ251.

[84] N. Becerra-Tomas, M. Guasch-Ferre, J. Quilez, J. Merino, R. Ferre, A. Diaz-López, M. Bulló, P. Hernández-Alonso, A. Palau-Galindo, J. Salas-Salvadó, Effect of functional bread rich in potassium, g-aminobutyric acid and angiotensin-converting enzyme inhibitors on blood pressure, glucose metabolism and endothelial function a double-blind randomized crossover clinical trial, Medicine (United States). 94 (2015) e1807. https://doi.org/10.1097/MD.0000000000001807.

[85] K. Inoue, T. Shirai, H. Ochiai, M. Kasao, K. Hayakawa, M. Kimura, H. Sansawa, Blood-pressure-lowering effect of a novel fermented milk containing gamma-aminobutyric acid (GABA) in mild hypertensives, Eur J Clin Nutr. 57 (2003) 490–495. https://doi.org/10.1038/SJ.EJCN.1601555.

[86] M. Shimada, T. Hasegawa, C. Nishimura, H. Kan, T. Kanno, T. Nakamura, T. Matsubayashi, Anti-hypertensive effect of gamma-aminobutyric acid (GABA)-rich Chlorella on high-normal blood pressure and borderline hypertension in placebo-controlled double blind study, Clin Exp Hypertens. 31 (2009) 342–354. https://doi.org/10.1080/10641960902977908.

[87] D. Espes, H. Liljebäck, H. Hill, A. Elksnis, J. Caballero-Corbalan, P.O. Carlsson, GABA induces a hormonal counter-regulatory response in subjects with long-standing type

1 diabetes, BMJ Open Diabetes Research & Care. 9 (2021). https://doi.org/10.1136/BMJDRC-2021-002442.

[88] F. Cavagnini, M. Pinto, A. Dubini, C. Invitti, G. Cappelletti, E.E. Polli, Effects of gamma aminobutyric acid (GABA) and muscimol on endocrine pancreatic function in man, Metabolism. 31 (1982) 73–77. https://doi.org/10.1016/0026-0495(82)90029-4.

[89] A.D. and A.M. F. Cavagnini, C. Invitti, M. Pinto, C. Maraschini, A. Di Landro, Effect of acute and repeated administration of gamma aminobutyric acid (GABA) on growth hormone and prolactin secretion in man, Eur J Endocrinol. 93 (1980) 149–154.

[90] T. Miyazawa, T. Kawabata, K. Okazaki, T. Suzuki, D. Imai, T. Hamamoto, S. Matsumura, T. Miyagawa, Oral administration of γ-aminobutyric acid affects heat production in a hot environment in resting humans, J Physiol Anthropol. 31 (2012) 3. https://doi.org/10.1186/1880-6805-31-3.

[91] T. Miyazawa, T. Kawabata, T. Suzuki, D. Imai, T. Hamamoto, T. Yoshikawa, T. Miyagawa, Effect of oral administration of GABA on temperature regulation in humans during rest and exercise at high ambient temperature., Osaka City Med J. 55 (2009) 99–108.

[92] A. Leonte, L.S. Colzato, L. Steenbergen, B. Hommel, E.G. Akyürek, Supplementation of gamma-aminobutyric acid (GABA) affects temporal, but not spatial visual attention, Brain and Cognition. 120 (2018) 8–16. https://doi.org/10.1016/J.BANDC.2017.11.004.

[93] A. YAMATSU, Y. YAMASHITA, I. MARU, J. YANG, J. TATSUZAKI, M. KIM, The Improvement of Sleep by Oral Intake of GABA and Apocynum venetum Leaf Extract, J Nutr Sci Vitaminol (Tokyo). 61 (2015) 182–187. https://doi.org/10.3177/jnsv.61.182.

[94] A. Yoto, S. Murao, M. Motoki, Y. Yokoyama, N. Horie, K. Takeshima, K. Masuda, M. Kim, H. Yokogoshi, Oral intake of γ-aminobutyric acid affects mood and activities of central nervous system during stressed condition induced by mental tasks, Amino Acids. 43 (2012) 1331–1337. https://doi.org/10.1007/s00726-011-1206-6.

[95] A.M. Abdou, S. Higashiguchi, K. Horie, M. Kim, H. Hatta, H. Yokogoshi, Relaxation and immunity enhancement effects of gamma-aminobutyric acid (GABA) administration in humans., Biofactors. 26 (2006) 201–8.

[96] W.-Y. Jung, S.-G. Kim, H.-K. Kim, J.-S. Lee, S.-I. Han, S. Choe, B.-G. Son, Effect of GABA Extract of Black Sticky Rice with Giant Embryo on Alcohol-Related Indices After Acute Alcohol Intake in Social Drinkers, Alcohol Clin Exp Res. 39 (2015) 1212–1218. https://doi.org/10.1111/acer.12746.

[97] P. Hannant, S. Cassidy, D. Renshaw, A. Joyce, A double-blind, placebo-controlled, randomised-designed GABA tea study in children diagnosed with autism spectrum conditions: a feasibility study clinical trial registration: ISRCTN 72571312,

Nutritional Neuroscience. 24 (2021) 45–61. https://doi.org/10.1080/1028415X.2019.1588486.

[98] H. Nakamura, T. Takishima, T. Kometani, H. Yokogoshi, Psychological stress-reducing effect of chocolate enriched with γ-aminobutyric acid (GABA) in humans: assessment of stress using heart rate variability and salivary chromogranin A, Int J Food Sci Nutr. 60 (2009) 106–113. https://doi.org/10.1080/09637480802558508.

[99] Y. Okita, H. Nakamura, K. Kouda, I. Takahashi, T. Takaoka, M. Kimura, T. Sugiura, Effects of vegetable containing gamma-aminobutyric acid on the cardiac autonomic nervous system in healthy young people., J Physiol Anthropol. 28 (2009) 101–7.

Chapter 2

Screening for GABA and glutamic acid in tomato and potato genotypes and effects of domestic cooking

Authors: Tessa H. de Bie[a, b], Ric C.H. de Vos[b], Henriëtte D.L.M. van Eekelen[b], Frank F. Millenaar[c], Cindy K.M. van de Wiel[d], Josephus J.H.M. Allefs[d], Michiel G.J. Balvers[a], Renger F. Witkamp[a], Maarten A. Jongsma[b]

Affiliations:

[a] Division of Human Nutrition and Health, Wageningen University & Research, P.O. Box 17 6700 AA Wageningen, The Netherlands

[b] Wageningen Plant Research, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, The Netherlands

[c]BASF's vegetable seeds business, Napoleonsweg 152, 6083 AB Nunhem, The Netherlands

[d]Agrico, Duit 15, 8305 BB Emmeloord, The Netherlands

Under revision at the Journal of Food Composition and Analysis

Abstract

γ-Aminobutyric acid (GABA) and its precursor glutamic acid play signaling roles in both humans and plants. Interestingly, positive effects on human health are ascribed to GABA consumption, which is present at relatively high levels in various food products, including potato tubers and tomato fruits. However, the currently available information on GABA content in foods only partly represents market categories and lacks data on glutamic acid. Here, we performed a screening of 98 tomato and 72 potato genotypes for GABA and glutamic acid levels. Our results show a large variation in both GABA and glutamic acid across the various genotypes. The GABA and glutamic acid levels ranged from 72 to 1122 µg/g fresh weight (FW), and 1160 to 6513 µg/g FW, respectively in tomato, and were between 68 to 759 µg/g FW and 409 to 874 µg/g FW in potato. Differences between market categories were only present for glutamic acid. For both GABA and glutamic acid, losses occurred with cooking, depending on the preparation. GABA was less affected by cooking than glutamic acid. Potato and tomato could be major dietary GABA sources. Especially high-GABA genotypes merit further investigation because of their potential health effects.

Keywords: GABA, glutamic acid, food, tomato, potato, screening, cooking, baking, frying

1. Introduction

γ-Aminobutyric acid (GABA) and its precursor glutamic acid are amino acids present at up to gram per kilo levels in many food products. They are either endogenously produced in for example melon, tomato and potato or generated during fermentation in for example matured cheese, fermented soy products and sauerkraut [1–6].

In plants, glutamic acid is incorporated into proteins and involved in amino acid metabolism as a precursor for (among others) GABA, and both molecules are involved in diverse physiological, defense, signaling and reproductive processes [7,8]. GABA is directly toxic to pest insects, and for this reason, it is presumed to be constitutively present in high amounts specifically in some fruits and storage organs like tomato and potato tubers (~500 µg/g) [9–11]. Yet, in tomato fruits, the high content of glutamic acid introduces an umami flavor which is assumed to enhance fruit consumption by birds and mammals [12]. This promotes seed dispersal as tomato seeds survive digestion and will germinate from fecal deposits.

In human biology, both glutamic acid and GABA are primarily known as neurotransmitters in the central nervous system. At the same time, oral supplementation with GABA has been shown to have metabolic health effects in rodents [13,14]. Most research on the health benefits of GABA in humans has been performed with supplemented GABA [15]. GABA supplements are widely available, claiming to improve sleep and reduce stress. Recently, we determined that GABA bioavailability is not influenced by a tomato food matrix [16]. This suggests that an increased intake of GABA, through the consumption of tomato fruits or potato tubers that are relatively high in GABA for example, could lead to positive health effects.

Tomato and potato are staple foods in many cultures. For instance, the consumption of fresh tomatoes in the EU is currently 15 kg per capita, and an additional 18 kg of processed tomatoes is consumed [17]. Potato is eaten in 82% of the world's countries and is one of the largest food energy suppliers. In 2013 the consumption of potatoes in the EU was 83 kg per capita, which is the largest volume in the world [18]. Consequently, they could provide a source of GABA in the diet, in particular when high GABA varieties are consumed and provided that preparation methods are compatible.

Next to GABA, the levels of glutamic acid are also relatively high in potato and especially in tomato [19]. It is relevant to study both GABA and glutamic acid in crops, considering their biosynthesis is interconnected by for example the GABA shunt [20]. While GABA is the most abundant free amino acid in green tomato fruits, its concentrations decrease substantially during ripening [21]. On the other hand, the free glutamic acid concentration increases steadily during tomato fruit ripening and it represents the most abundant amino acid in red tomato fruits. Research investigating glutamic acid content of tomato fruits is mainly focused on increasing the characteristic umami flavor to which glutamic acid contributes [22].

Currently, there is considerable interest in increasing the GABA content of food products. A gene-edited tomato containing 1250 µg/g FW GABA is already marketed in Japan [23]. In addition, relatively GABA-rich plant products can be obtained by specific growth conditions (e.g., salt stress [24]), selection among existing cultivars, or by means of breeding programs making use of the genetic variation in wild species [25]. Some literature describing the GABA and glutamic acid content of different tomato and potato genotypes is already available. For GABA in tomato fruits, a screening of 61 genotypes was performed in a field trial by Saito et al. (2008), who found a 20-fold difference between genotypes in GABA content (ranging from 90 to 1000 µg/g) [5]. For glutamic acid only a narrow selection of tomato genotypes has been investigated and the content range was not more than 2-fold [26–28]. For example, Pratta et al (2004) studied the glutamic acid content in 5 different tomato cultivars and reported a variation from 1600 to 2800 µg/g FW [26]. For potato, Nakamura et. al (2006) analyzed the GABA content of tubers from 22 varieties and showed a range of about 3.5 fold, from 239 to 819 µg/g FW [6]. However, these genotypes only partly represent the existing market categories and none of these screenings involved both GABA and its precursor glutamic acid [6,29–32]. Thus, a large representative screening of both GABA and glutamic acid in tomato or potato is still lacking [6,30–33].

Therefore, we aimed to determine the extent of variation in GABA levels in a representative range of tomato and potato genotypes. In the search for tomato and potato varieties with a high GABA content, we analyzed the levels of GABA and its precursor glutamic acid in 98 tomato and 72 potato genotypes and cultivars. To gain more insight, we also studied the variation in effect of fruit maturation on GABA content and the effect of harvest year. As tomato is frequently consumed after some form of processing and potatoes are not eaten raw, we also included an analysis of the effects of most commonly used domestic cooking methods on GABA and glutamic acid content.

2. Methods

2.1. Planting, harvesting and sampling procedures

2.1.1. Potato

A series of 72 genotypes, consisting of cultivars and breeding clones, were grown in clay soil in the seasons of 2017, 2018, and 2019 in Bant, Noordoostpolder, The Netherlands. Seed tubers had been produced and stored at that location in the previous year. The planting dates were in April on the 12th, 18th, and 17th in the three consecutive growing seasons, respectively. Plots consisted of 16 plants each, planted in two rows that were 75 cm apart. Planting was at a 33 cm distance within rows, with 4 border plants separating the plots of different genotypes. Fertilization was in total 180 to 190 kg of N per ha, including the soil stock of approximately 25 kg N per ha. Of this total N, 120 kg per ha was given in the form of a N:P:K mixture in a ratio 23:23:0 immediately after seed tuber planting, but before hilling, and the remaining N was supplied in the form of calcium ammonium nitrate after tuber initiation of the mature plants. Protection against plant diseases was carried out according to local agricultural practice. Harvest dates of mature tubers were October the 16th in 2017 and September the 12th and 19th in 2018 and 2019, respectively. Tubers were mechanically harvested and subsequently stored in boxes at ambient temperature (8°C to 12°C) in the dark for about 3 months until sampling for analysis.

Ten average-sized tubers were randomly chosen per box and washed with tap water. One wedge, approximately 0,5 cm width at its widest side, was cut from each tuber between the stolon and stem end. Wedges were quickly cut into smaller parts and frozen in liquid nitrogen for immediate processing or storage at -80°C. For a range of genotypes (Cumbica, Monika, Concordia, AR 12-5137, Hansa, Cerisa, Blue Star, Fontane, AR 91-1409, ARD 11-3181) the GABA and glutamic acid content was also assessed in peel and flesh separately. For this experiment, 10 tubers per genotype were selected. The cut wedges of 0.5 cm wide were peeled, the flesh was cut into small pieces and both flesh and peel were stored and processed separately. Tuber parts were then milled into a fine powder using a liquid nitrogen-cooled analytical mill (IKA, Staufen, Germany) and aliquots (100 mg, < 2.5% deviation) of the frozen ground powders were weighed in a 2 ml Eppendorf tube) and stored in 4 ml cryotubes at -80 °C until analysis.

2.1.2. Tomato

A collection of 98 tomato genotypes, consisting of both cultivars and breeding lines, was screened. The cultivars represented both cluster (normal round and beef), snack, and cherry types of fruits. The breeding lines partly consisted of heirloom genotypes and elite lines. Seeds were sown in small rockwool plugs (Oct 27, 2017), transplanted in blocks (Dec 10, 2017) and finally transplanted to a greenhouse with rockwool slabs (Jan 4, 2018). The greenhouse was located in Roggel (Netherlands). Pruning of the trusses from plants in the cluster segment was done to 6 fruits remaining per cluster.

Only fully ripe fruits were used in the genotype screening. Harvest started on March 14, 2018, and the fruits used for the measurements were harvested on May 21, 2018.

The effect of fruit ripening on GABA and glutamic acid levels was subsequently evaluated in 7 other tomato genotypes (cv. Madara, Marejada, Maremagno, Marinda, Marmarino, Micro Tom and Moneymaker). For this experiment, seeds were sown on December 14, 2018 and May 15, 2020, transplanted in blocks on January 3, 2019 and May 29, 2020, and finally transplanted in the greenhouse on January 31, 2019 and June 18, 2020, respectively. Mature green, breaker, turning, ripe and overripe fruits were harvested. The greenhouse was located in Horst (Netherlands). No cluster pruning was applied in this experiment.

The growth conditions were comparable to commercial tomato growing conditions in the Netherlands. The nutrient solutions provided had an electrical conductivity of 2,2 mS/cm. Immediately after harvesting, the fruits were transported to Wageningen where the samples were processed the same or next day. Per genotype/ripening stage 10 representative fruits were randomly selected. From each tomato, a wedge (top to button) with the size of 1/8 of the fruit was cut with a sharp knife and quickly frozen in liquid nitrogen in pools per genotype. The frozen wedges were ground into a fine powder per genotype using a liquid nitrogen-cooled grinder (IKA).

2.2. GABA and glutamic acid measurements

The levels of GABA and glutamic acid present in the tomato and potato samples were quantified using gas chromatography coupled to mass spectrometry (GCMS). The extraction and analysis procedures were mainly according to the protocol described previously [33]. Briefly, 100 mg (< 2.5% deviation) of the frozen ground tomato or potato powder was weighed into a 2 ml Eppendorf tube. The powders were extracted with 1400 µL of a 90% (v:v) methanol/water solution containing 15 µg/mL of GABA-d6 and 50 µg/mL of L-glutamic acid-d5 (both from Merck-Sigma, Zwijndrecht, The Netherlands) as deuterium-labelled internal standards. After 10-minutes of sonication followed by 10-minutes of centrifugation, 500 µL of the clear supernatant was taken and mixed with 375 µL of chloroform and 750 µL of water. After a new centrifugation step, 50 µL aliquots of the upper (polar) phase were transferred into a glass insert placed in an open 2 mL GC vial. Extracts were dried overnight (16 h) by vacuum centrifugation (Savant®, SPD121P, Thermo Scientific, Breda, The Netherlands) at room temperature, and the vials were subsequently closed under an argon atmosphere with magnetic crimp caps. Prior to their analysis, dried extracts were derivatized online using a TriPlusRSH autosampling/injection robot (Thermo Scientific), which was programmed to firstly add 17.5 µL of O-methylhydroxylamine hydrochloride (20 mg/mL pyridine) (both from Merck-Sigma, Zwijndrecht, The Netherlands), then incubated for 30 min at 40 °C with agitation, and finally derivatized the compounds with 17.5 µL of N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA))(Merck-Sigma, Zwijndrecht, The Netherlands), for 60 min at 40 °C. After derivatization, compounds were separated on a Trace 1300 gas chromatograph system (Thermo Scientific). Of each sample 1 µL was injected onto a PTV injector (70 °C). By using a split flow of 19 mL/min samples were introduced onto a VF-5 ms capillary column + 10 m guard

column (30 m × 0.25 mm × 0.25 µm, Agilent Technologies) for chromatographic separation. Helium (5.0) was used as carrier gas at a constant column flow rate of 1 mL/min. The temperature program of the GC oven started at 70 °C (2 minute hold) and rose with 10 °C/min to 310 °C (with 10 minute hold). Separated compounds were ionized by electron impact at 70 eV and mass spectra were acquired at full scan mode with a m/z range of 50 to 600 at an ion source temperature of 290 °C using a TSQ8000 DUO-series triple quadrupole mass spectrometer (Thermo Scientific). A dilution series of 0.1 to 500 µg/ml of both GABA and glutamic acid was used to quantify these compounds in the samples, while correcting for the recovery of their deuterated standards. Representative chromatograms are presented in supplementary material 1.

In view of the large numbers of samples, we chose for repeated extraction and analysis of a pool of powders from randomly chosen potato or tomato genotypes, rather than analyzing each individual powder in two or more technical replicates. This pool of powders was extracted 11 times in the same manner as the individual powders and analysed by GCMS before, after and throughout the series of the real samples. Based on these quality control samples (QCs), we were able to calculate the overall analytical variation, which was 5.8% for GABA and 16.2% for glutamic acid.

2.3. Domestic cooking methods

Single batches of potato tubers (cv. 'Agria', provided by Agrico Research) and tomato fruits (cv. 'Trevine', provided by Nunhems) were prepared with different domestic cooking techniques (Tables 1 and 2) to assess the effects on GABA and glutamic acid content. A laboratory sample consisted of 10 randomly selected tubers or fruits. All operations using raw samples were performed swiftly in order to prevent any degradation of the sample. Each cooking method was performed in triplicate on different days. After preparation and cooking, samples were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Samples were ground to a fine powder using a liquid nitrogen-cooled grinder (IKA). Analysis of GABA and glutamic acid content was performed as described in sections 2.2. In order to correct GABA and glutamic acid levels for potential differences between preparation techniques due to differential moisture losses, the pre- and post-treatment dry matter content was determined from their ground powders, according to the official method recommended by the AOAC [34].

Table 2: Preparation methods before cooking and cooking conditions for each cooking method applied to potato

Cooking technique	Preparation	Cooking conditions
Raw	Peeled	-
Boiling	Peeled, 2 cm thick slices	100 °C for 10 min in water with a ratio of 1:3 (potato:water, v:v)
Frying	Peeled, 1 cm x 1 cm x length potato (French fry shaped)	6 min at 150 °C in sunflower oil, cooled, for 4 min at 175 °C in sunflower oil
Steaming	Peeled, 2 cm thick slices	Steamed for 20 min in a steam oven (RATIONAL SelfCookingCenter)
Baking	Peeled, 1 cm thick slices	Baked in a hot-air oven (RATIONAL SelfCookingCenter) for 15 min at 200 °C with 2 tbsp of oil
Open pan frying	Peeled, 5 mm thick slices	Baked on one side for 5 min and for 2.5 min on the other side at medium-high temperature in 2 tbsp. oil
Microwave cooking	Peeled, 2 cm thick slices	900W for 11 min in microwave (Panasonic Pro II Type NE-1880) with 2 tbsp of water

Table 3: Preparation method before cooking and cooking conditions for each cooking method applied to tomato

Cooking technique	Preparation	Cooking conditions
Raw	Whole fruits	-
Boiling	Whole fruits	15 min at 100 °C in water (tomatoes were submerged in water)
Baking	Whole fruits	Baked in a hot-air oven (RATIONAL SelfCookingCenter) for 15 min at 200 °C

2.4. Data analysis

Statistical analyses were performed using the R environment for statistical computing version 4.0.2 (https://www.R-project.org) and IBM SPSS Statistics version 25. To assess significant differences between market categories, an ANOVA was performed with a Tukey post hoc adjustment for multiple comparisons. An independent samples t-test was performed to assess differences between different cooking techniques and differences between peel and flesh. An ANOVA and ANCOVA with Bonferroni correction was used to assess differences in GABA and glutamic acid content over different ripening stages with genotype as a covariate. The correlations between compound levels over different years and between GABA and glutamic acid across samples were assessed using Pearson's correlation.

3. Results

3.1. Variation in GABA and glutamic acid levels across tomato and potato genotypes

3.1.1. Tomato

Contents of GABA and glutamic acid were determined for 98 different tomato genotypes and results are shown in figures 1 and 2, respectively. Categories were either cherry, cluster, heirloom, or snack tomatoes. On average, the tomato genotypes contained 242 ± 89 µg/g FW GABA, in a range from 72 to 558 µg/g FW. This corresponds with an 8-fold difference between the highest (found in a heirloom tomato fruit) and lowest (found in a cherry tomato fruit) accumulators of GABA. A one-way ANOVA revealed that there was no statistically significant difference in GABA content between the 4 market categories ($F(94, 3) = 1.92$, $p = 0.13$).

For glutamic acid, the average content was 3694 ± 1156 µg/g FW in a range from 1160 to 6513 µg/g FW. This resulted in a 6-fold difference between the highest (found in a cherry tomato fruit) and lowest (found in an heirloom tomato fruit) accumulators of glutamic acid. In this case, a one-way ANOVA showed that there was a statistically significant difference in glutamic acid content between the market categories ($F(94, 3) = 17.70$, $p = <0.001$). Tukey's HSD Test for multiple comparisons showed that the glutamic acid content of the cherry tomatoes (4649 ± 925 µg/g FW) was significantly higher than in the other market categories. No correlation was found between the GABA and glutamic acid content across the tomato fruits analyzed (Supplementary material 2).

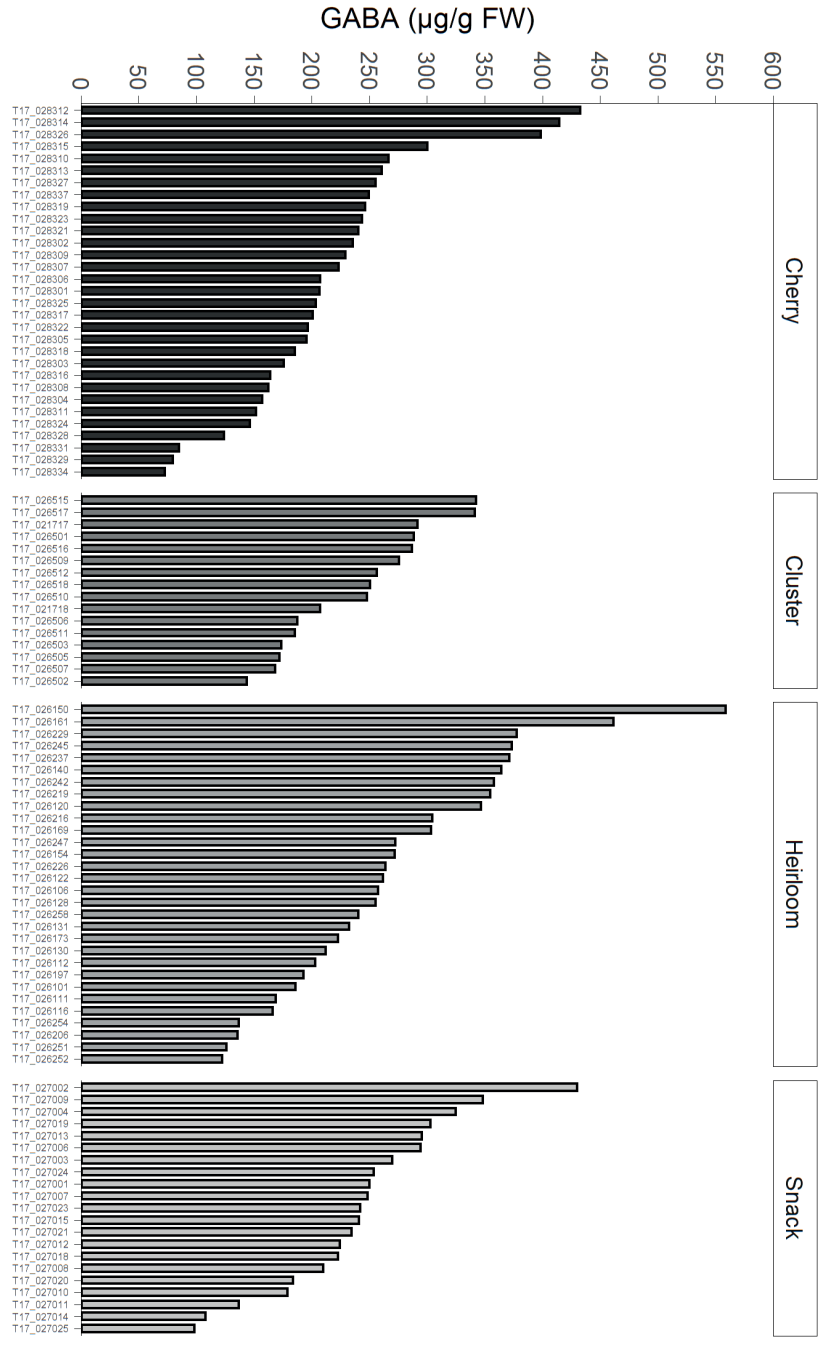


Figure 1: GABA content per gram fresh weight of different tomato genotypes divided in cherry, cluster, heirloom and snack tomatoes. Genotypes are ordered from high to low GABA levels per fruit type. Each data point represents a pooled sample of 10 tomato fruits.

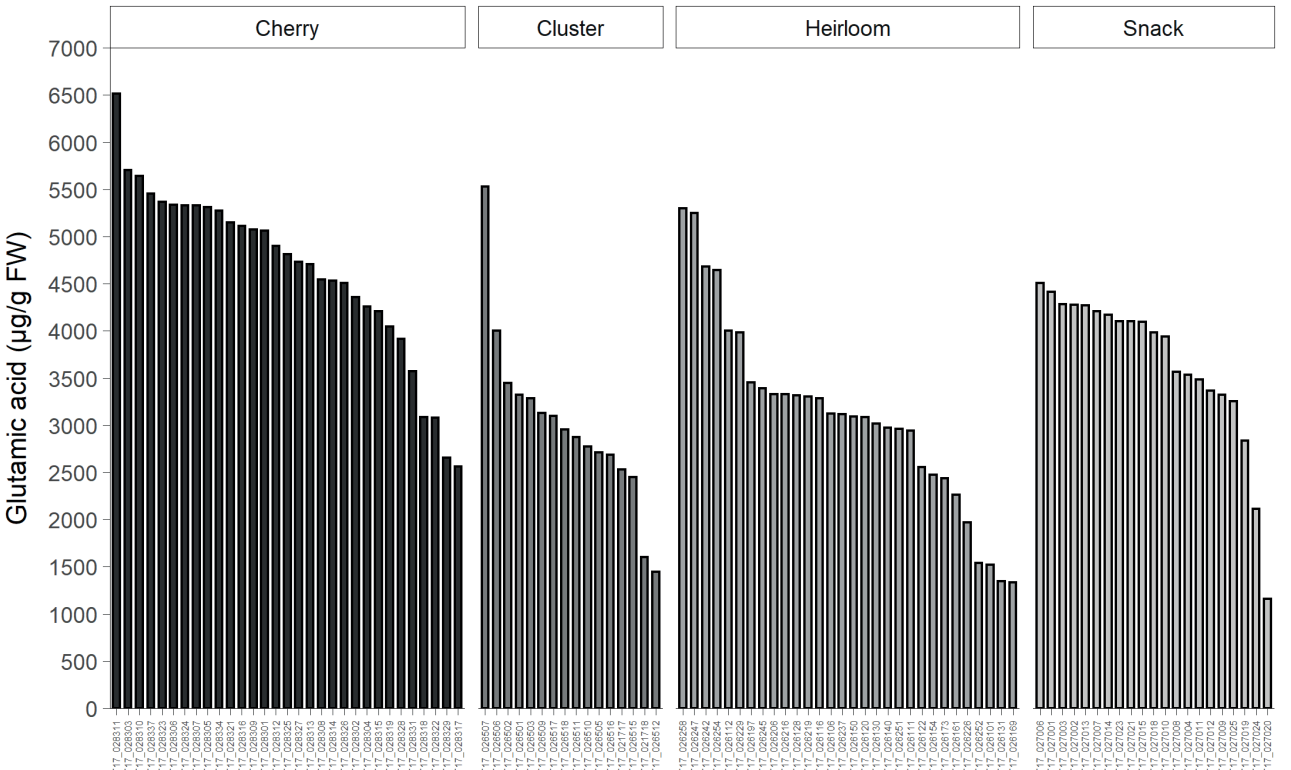


Figure 2: Glutamic acid content per gram fresh weight of different tomato genotypes divided in cherry, cluster, heirloom and snack tomatoes. Genotypes are ordered from high to low glutamate levels per fruit type. Each data point represents a pooled sample of 10 tomato fruits.

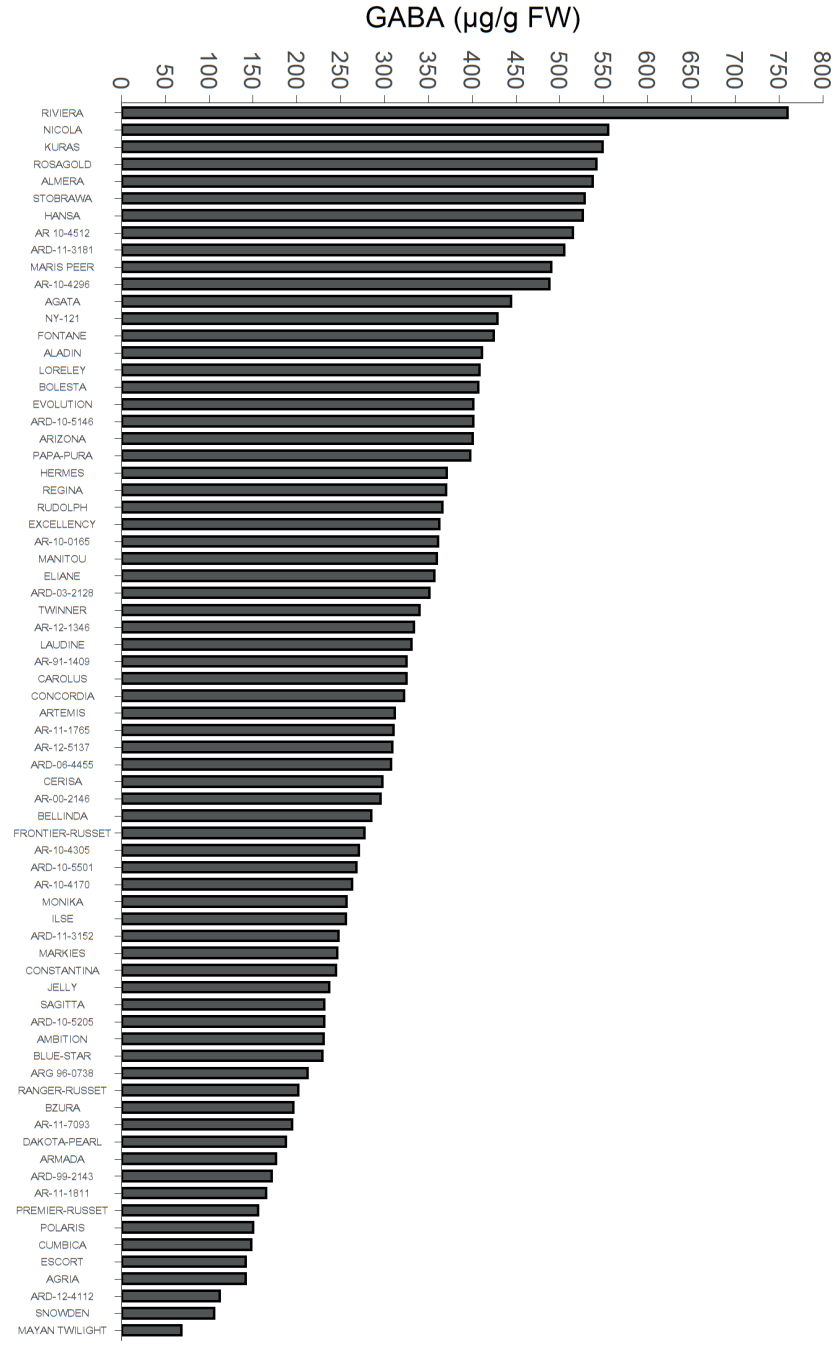


Figure 3: GABA content per gram fresh weight of different potato genotypes. Each data point represents a pooled sample of 10 potato tubers.

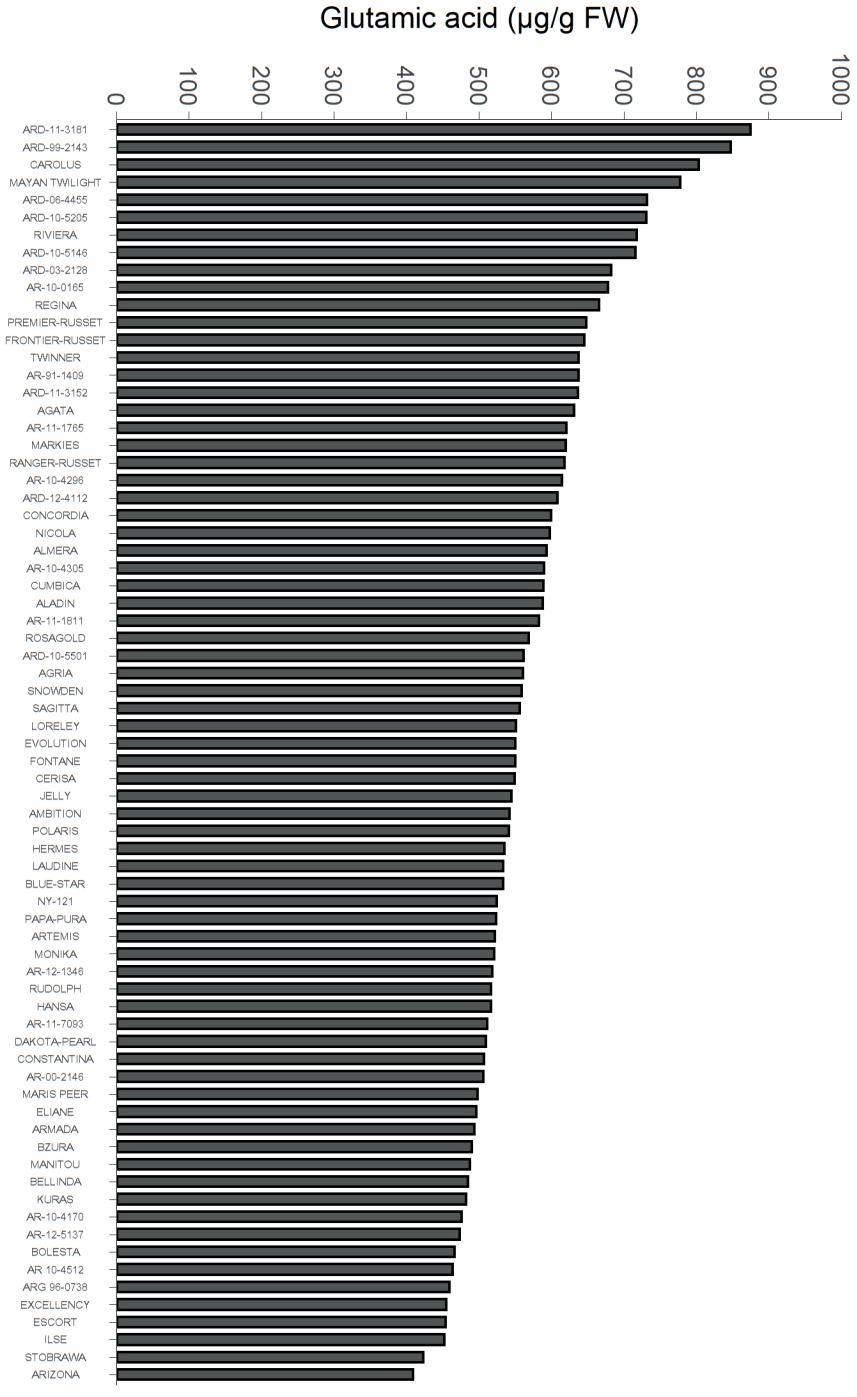


Figure 4: Glutamic acid content per gram fresh weight of different potato genotypes. Each data point represents a pooled sample of 10 potato tubers.

3.1.2. Potato

The GABA and glutamic acid content was measured in 72 different potato cultivars and breeding clones. These represent a wide variety of market categories such as fresh retail category (e.g. Rosagold, Loreley), processing for fries (e.g. Fontane, Markies), for crisps (e.g. Hermes, Snowden), for starch extraction (Kuras), diploid cultivar group Phureja (Mayan Twilight, Papapura), Dutch germplasm (Armada, Artemis), Polish germplasm (Bzura, Strobrawa), UK germplasm (Maris Peer), German germplasm (Jelly, Regina) and USA germplasm (Premier Russet, Dakota Pearl). On average, these potato tubers contained 321 ± 132 µg/g FW GABA in a range from 68 to 759 µg/g FW (figure 3), while for glutamic acid the average was 571 ± 97 µg/g FW in a range from 409 to 874 µg/g FW (figure 4). Therefore, an 11-fold variation was observed for GABA and a 2-fold variation for glutamic acid. The cv. 'Riviera' stood out with a 33% higher GABA content than all other cultivars and breeding clones. Like in tomato fruit, no correlation was found between the GABA and glutamic acid content in potato tubers (Supplementary material 3).

3.2. The variation in effect of harvest year on GABA levels in tomato and potato and fruit maturation on GABA levels in tomato

3.2.1. Tomato

As differences in GABA content were observed in ripe tomato fruits, we analyzed a second series of genotypes that were harvested the same year (either 2018/2019 or 2020) but at different stages of their ripening. We aimed to determine what the relevance is of harvesting moment for the GABA content. For all genotypes, a decrease in GABA and an increase in glutamic acid was observed during fruit ripening (figure 5). . On average the GABA content is significantly higher in green tomatoes as compared to all other stages and the glutamic acid content is significantly higher in overripe tomatoes as compared to the green and breaker stages. Genotype was only of significant influence on the relationship between ripening stage and GABA content ($p = < 0.001$) and not on the relationship between ripening stage and glutamic acid content ($p = 0.08$). Proportionally, the difference in GABA content between ripe and green tomato fruits is smallest in the Madara cultivar, while the difference in glutamic acid content is largest in this cultivar. The cv. 'Madara' also had an exceptionally high GABA level of 1122 ± 104 µg/g FW in its ripe fruits. We subsequently investigated the reproducibility of this relatively high GABA content in Madara, over different harvest years (figure 6). A one-way ANOVA showed that GABA content was not different between the different years ($F(25, 1) = 0.53$, $p = 0.47$), suggesting a constitutive relatively high level in 'Madara'.

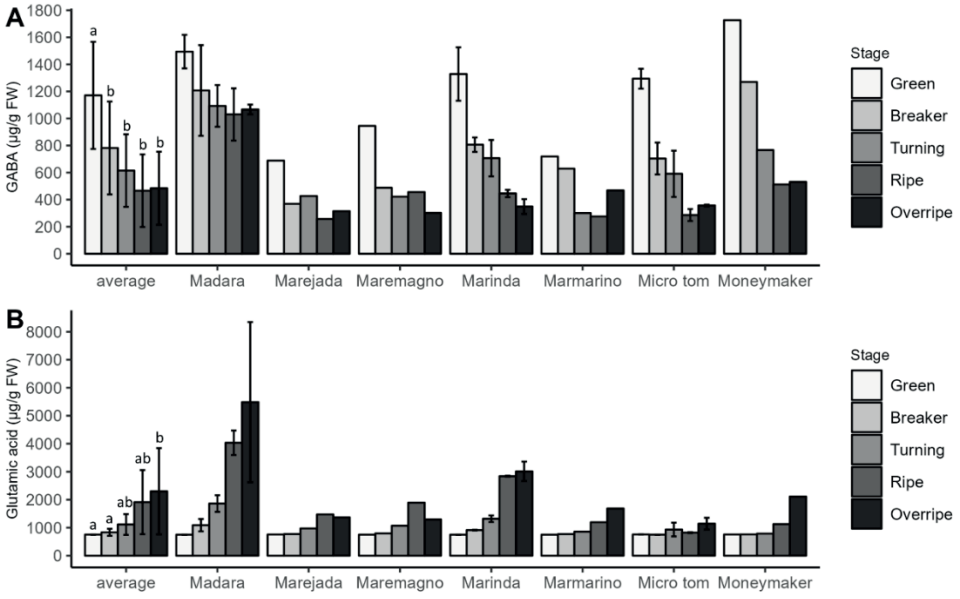


Figure 5: A) GABA and B) glutamic acid content in 7 different tomato genotypes, over different stages of ripening (Green, Breaker, Turning, Ripe and Overripe). Each data point represents a pooled sample of 10 tomato fruits. A second harvest (same year, different dates) of cv 'Madara', 'Marinda' and 'Micro tom' was analyzed to assess batch variation in GABA and glutamic acid levels. For these 3 cultivars the data is presented as mean ± SD of the two replicates. The barplot also shows the overall average of all 7 genotypes with letters indicating significant differences between ripening stages.

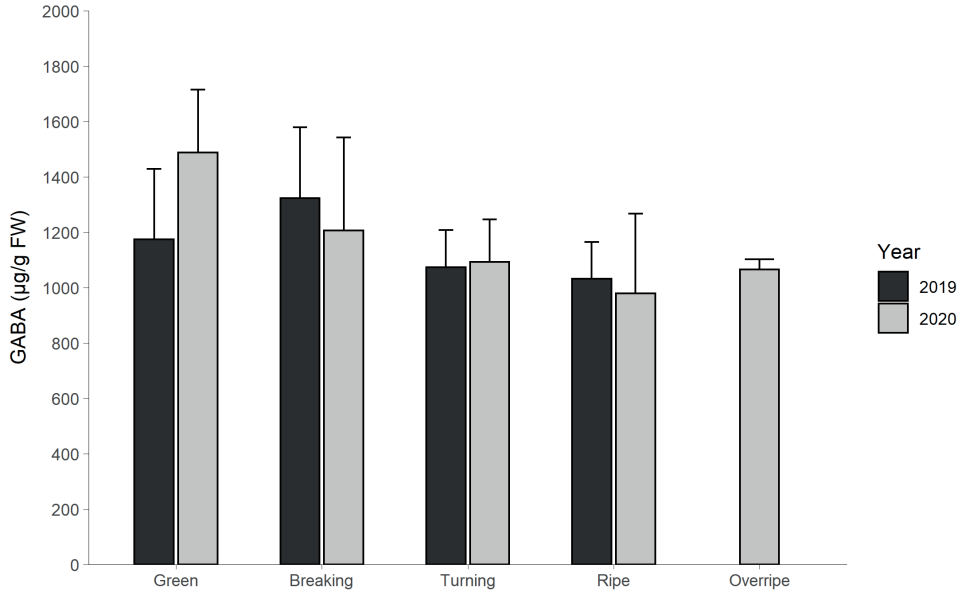


Figure 6: GABA content measured in cv. 'Madara' over different stages of ripening in 2019 and 2020. Data are presented as mean and standard deviation. Replications represent different harvesting dates, on each date a pooled sample of 10 tomato fruits was sampled per ripening stage. n=3 in 2019, n=3 for the green and ripe stage in 2020 and n=2 for the other stages in 2020.

3.2.2. Potato

We also assessed the reproducibility of the GABA content in potato genotypes across years. In a selection of 22 genotypes from the original screening in 2018 (figure 3 and 4), the GABA content was again determined in tubers from the harvest in 2019 (figure 7) and for an additional 9 potato genotypes the GABA content was measured in tubers from the harvest in 2020. The tuber GABA content in 2019 and 2018 was highly correlated across similar genotypes ($r = 0.87$; 95% CI 0.70, 0.94; $P < 0.001$); also the levels obtained from harvest 2020 were highly correlated with those from harvest 2018 ($r = 0.94$; 95% CI 0.72, 0.99; $P < 0.001$).

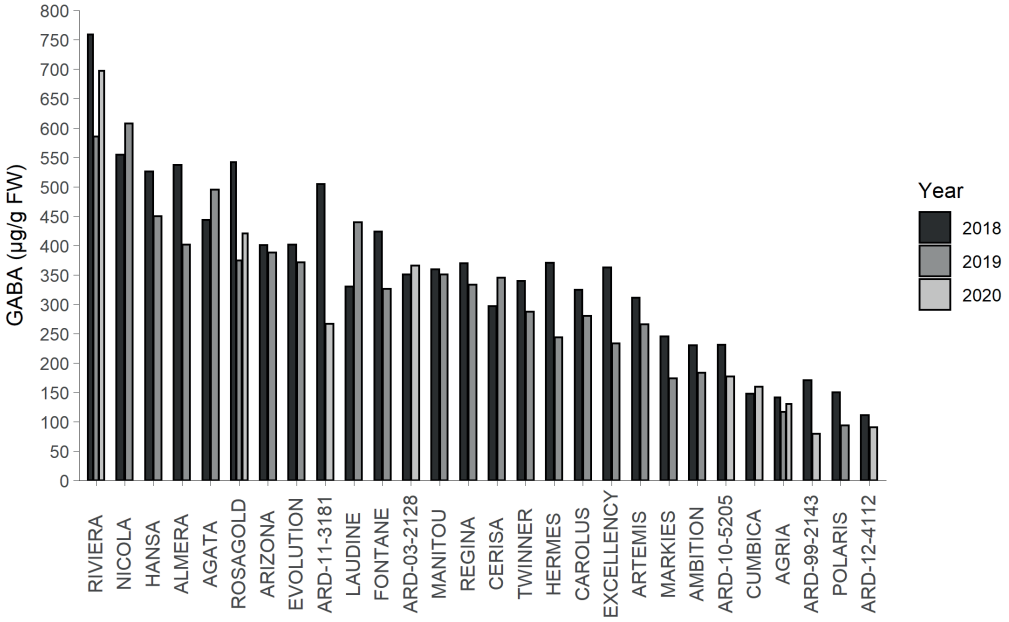


Figure 7: Reproducibility of GABA content per gram fresh weight for potato genotypes grown in 2019 (n=22) and/or 2020 (n=9) as compared to tubers from 2018. Each value represents a pool of 10 potato tubers.

3.3. Effect of domestic cooking methods on GABA and glutamic acid levels

Preparation of tomatoes and particularly potatoes frequently involves heat and other food-handling activities before their consumption. These cooking methods potentially affect the levels of GABA and glutamic acid in the consumed food products. To determine their impact, frequently employed potato and tomato cooking methods were applied and assessed for their effects on GABA and glutamic acid content. In these experiments, the compound levels were calculated on a dry weight basis, to account for potential differential water loss during product preparation. Table 3 shows the GABA and glutamic acid content per gram dry weight expressed as a percentage of the initial raw product. Dry weight measurements and GABA and glutamic acid content per gram of fresh weight are given in supplemental materials 4 and 5. As indicated in Table 3, in potato, most cooking methods resulted in considerable (12-52%) loss of glutamic acid. Also in tomato, boiling resulted in a significant (35%) loss of glutamic acid. Glutamic acid losses after baking tomatoes were more variable between replicate experiments (possibly due to differences in bursting of fruits). In potato, GABA levels were also significantly reduced after frying, steaming and microwaving, although to a lesser extent than glutamic acid (17% to 19%). Boiling of potatoes also tended to reduce their GABA content, while no effect was observed with baking and pan-frying. In contrast to its decreasing effect in potato, boiling tended to increase the GABA

content in tomatoes (42%, P=0.06), while baking the tomatoes did not alter their content.

Table 3: Effects of different cooking methods on GABA and glutamic acid content based on dry weight and expressed as percentage of the raw sample. Cooking methods were replicated on 3 different days, each time a pooled sample from 10 fruits or tubers was taken.

Method	GABA[1] (% of raw)	Glutamic acid[1] (% of raw)
Potato		
Raw	*100 (7.0)*	*100 (2.6)*
Boiled	*74.5 (19.2)·*	*88.5 (6.7)·*
Fried	*82.9 (2.1)**	*47.8 (2.1)****
Steamed	*81.3 (8.8)**	*88.2 (5.8)**
Baked	*98.8 (7.3)*	*72.0 (6.7)***
Pan-fried	*84.1 (26.4)*	*71.9 (19.6)·*
Microwaved	*81.7 (3.6)**	*77.1 (7.8)***
Tomato		
Raw	*100 (20.4)*	*100 (16.6)*
Boiled	*141.5 (18.7)·*	*64.7 (9.2)**
Baked	*99.2 (10.9)*	*53.3 (25.5)·*

*[1] Data is presented as mean (SD) percentage of the raw potato or tomato sample. Significant and trend differences are shown as compared to the raw potato or tomato sample, as assessed with an independent samples t-test. ·: P<0.10; *: P<0.05; **: P<0.01; ***: P<0.001.*

To study these effects of various domestic cooking methods, peeled potatoes were used. In order to determine the distribution of both GABA and glutamic acid between peel and remaining tuber (flesh), we separately analyzed peel and flesh tissue from tubers of 10 different potato genotypes for both GABA and glutamic acid content (figure 8). The difference in GABA content between the peel and flesh varied between genotypes, while no significant difference was observed on average. In contrast, the glutamic acid content was consistently and significantly higher in the peel than in the flesh.

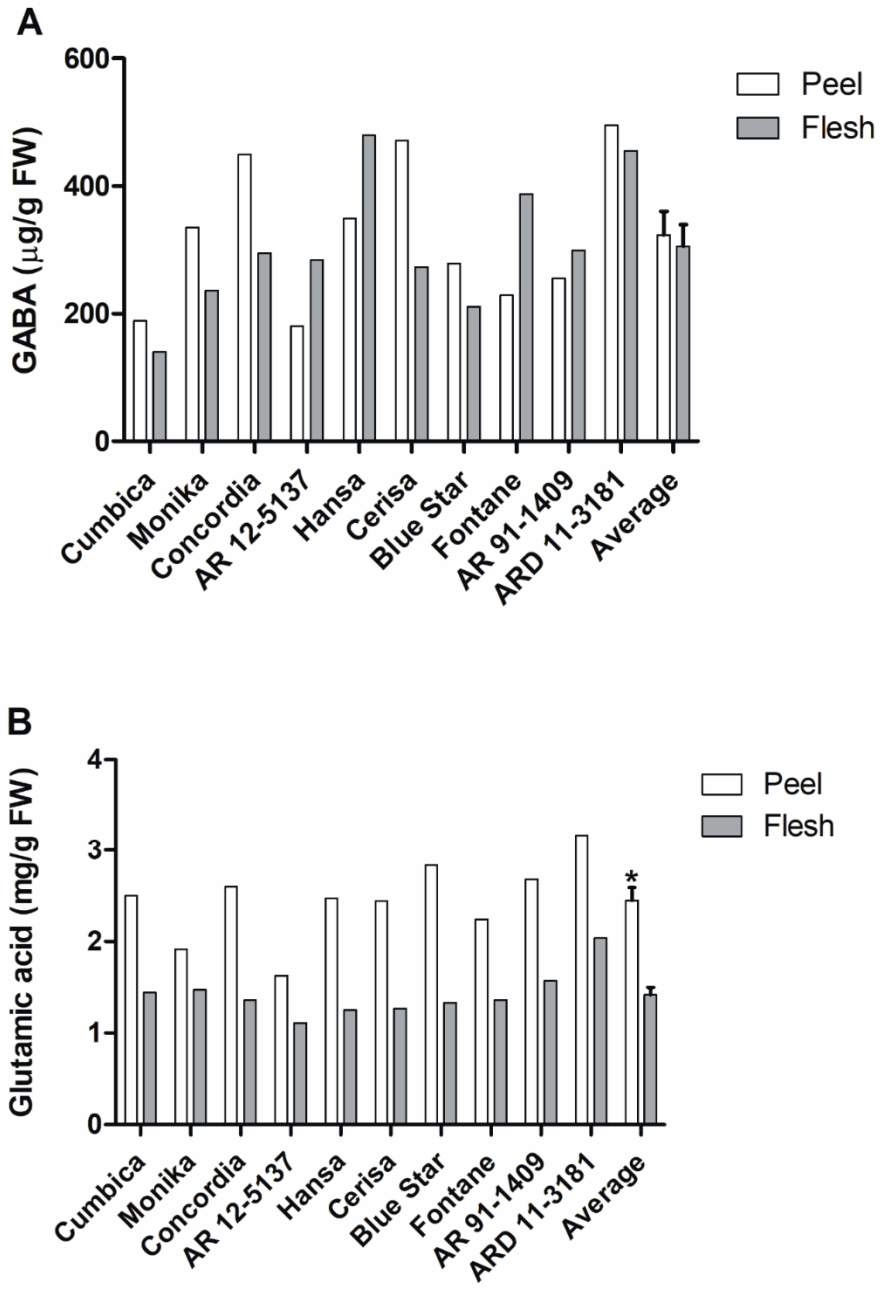


*Figure 8: Comparison of A) GABA and B) glutamic acid content between peel and flesh. Values are shown from pooled samples of 10 tubers. As a last bar, averages of the peel and flesh of the presented genotypes are shown as mean and SD. * indicates a significant difference.*

4. Discussion

In search of tomato and potato varieties with a relatively high GABA content, a large selection of 98 tomato and 72 potato genotypes was analyzed. The concentration of both GABA and its precursor glutamic acid was assessed and substantial natural variation was found to be present in GABA (11- and 14-fold resp.) and glutamic acid (2- and 6-fold resp.) levels in both potato and tomato. Although glutamic acid is the direct precursor in the biosynthesis of GABA, their levels do not correlate across genotypes analyzed. In ripening tomato fruit, the GABA content generally decreases while glutamic acid content increases. Interestingly, the cultivar containing the highest GABA level in ripe fruits, i.e. cv. 'Madara', also retained the most GABA during ripening. In addition, the GABA content of both tomato (only assessed for Madara) and potato (various genotypes) was found to be reproducible across different growing seasons. Specific genotypes of tomato (e.g. Madara with ~1000 ug/g) and potato (e.g. Riviera with ~700 ug/g) could therefore be a relevant source of GABA in the human diet, especially because GABA was found to be largely tolerant to diverse commonly used domestic cooking methods (at most 25% decrease). In contrast, the glutamic acid content was found to decrease more as a result of domestic cooking.

The genetic variation in GABA content between tomato genotypes (including cv. 'Madara') as reported in the current paper, is similar to that previously found in other studies. A range from approximately 90 to 1000 µg/g FW was observed in a similarly large screening of tomato genotypes [5]. The GABA content in potato has not been screened extensively before. Nakamura et. al (2006) found a GABA content ranging from 239 to 819 µg/g FW in 22 potato genotypes [6]. The rangeobserved is larger and also includes genotypes with a lower GABA content, most likely because more genotypes were included. The lowest level observed in potato was 68 µg/g FW.

Literature suggests that the levels of GABA and its biosynthetic precursor glutamic acid are linked [35], although this hypothesis has not been assessed across a wide range of genotypes. In the current study, no clear correlation between GABA and glutamic acid levels across genotypes was found, neither in mature tomato nor in potato. This result suggests that breeding towards a higher GABA content does not automatically lead to changes in glutamic acid content. This is especially important for tomato fruits, in which free glutamic acid is one of the main compounds responsible for its "umami" taste [27].

In this study, the potential causes of the observed natural variation in GABA content was not investigated. GABA is primarily produced by glutamic acid decarboxylase (GAD) from glutamic acid and catabolized by GABA transaminase (GABA-T) into succinate [36]. GABA content is, therefore, likely determined by the activity of these two enzymes. Indeed, targeted mutagenesis in the GAD gene effectively increases the GABA content in tomato, reaching levels up to 1250 µg/g FW [23]. In the current study, cv. 'Madara' appeared to be able to accumulate levels up to ~1000 µg/g FW, which is in the same range as the GAD-gene-edited tomato. However, for reliable quantitative comparisons it will be necessary to grow these gene-edited and natural genotypes together under the same conditions, harvest them at the same ripening stage and analyze them with the same analytical methods.

In general, a decrease in GABA content and an increase in glutamic acid content was observed during the tomato fruit ripening process. Previously, it was shown that the GABA content of fruits from cv. 'Micro Tom' decreases about 6-fold from the mature green to the red ripe stage of their ripening [35]. A similar 5-fold decrease in this cv. 'Micro Tom' was observed. A decrease in the GABA content of the high-GABA cv. Madara was also observed, but this decrease was relatively small (1.5-fold) compared to that in both Micro Tom and to other cultivars. Similar to the GABA-rich *DG03-9* variety described by Saito et al. (2008), Madara also seems to lose less GABA during ripening [5,35]. Such relatively low loss is possibly due to a differential ripening-related regulation of either GAD activity or GABA-T activity, or both, as compared to other genotypes [12]. Other than the *DG03-9* variety, Madara also seems to accumulate glutamic acid more and earlier in the ripening process compared to other genotypes. Future studies focusing on this differential regulation may provide more insight into the mechanism(s) behind a relatively high GABA level in ripe tomato fruit. Crossing these two high-GABA varieties might help to explore any genetic synergy and to produce offspring with even higher GABA levels.

The GABA content was reproducible across different growing seasons, as was observed for tomato cv 'Madara' and a range of potato genotypes. In contrast, Saito et al. (2008) showed poor reproducibility of tomato fruit GABA content over years [5]. However, in contrast to the current study, the tomato plants of Saito et al. (2008) were grown in the field. Since GABA content can be influenced by environmental stressors (Bown and Shelp, 2016; Kinnersley and Turano, 2010), it is well possible that differences in growth conditions between years could have caused this discrepancy. The standardized greenhouse growth conditions for tomato, as used in the current study, may better ensure a reproducible GABA content (only 9.7% difference within genotype between years on average) than field conditions.

Tomatoes are often processed before their consumption and potatoes are never eaten raw. Therefore, the effect of popular domestic cooking methods on the GABA and glutamic acid levels in tomato and potato was also investigated. Consistent with the literature, GABA appeared to be relatively more resistant to most of the preparation processes than glutamic acid (Li et al., 2017; Ma et al., 2022). Frying, steaming, and microwaving potatoes decreased the GABA content by ~20% in potatoes, while also boiling tended to decrease GABA. While in tomato no decrease in GABA was observed by any treatment, glutamic acid was significantly lowered by boiling and baking. In the experiments described in this manuscript, the tomatoes were boiled as whole fruits including peel. Removing the peel may potentially lead to more losses, as both GABA and glutamic acid are potentially able to dissolve into the boiling water. For potato the peel was removed from the tubers before cooking. Preparing the potatoes with the peel still present thus could possibly have prevented the observed GABA losses. Interestingly, boiling tended to increase the GABA content in tomatoes by approximately 40%, whereas the glutamic acid content was decreased by 35%. GABA is known to accumulate in response to several abiotic stress conditions like cold, heat, and salt [37]. Prolonged heat-drying of green soybeans up to a temperature of 40 degrees increased GABA content by a factor 5 for example [40]. Thus, a possible explanation for the observed increased GABA level in boiled tomatoes is an (temporarily) ongoing or even increased GAD activity upon heating. Further research

on the effect of moderate heat treatment on GABA accumulation might lead to a recommendation of slow cooking as an appropriate processing method to additionally increase GABA in intact tomatoes and possibly also in intact, non-peeled potatoes.

The presented effects of domestic cooking were calculated on dry weight, to take into account the difference in water losses between cooking methods. From a nutritional perspective, the GABA and glutamic acid content should also be considered per gram of fresh weight and portion size. For example, frying results in substantial water loss, which concentrates the GABA and glutamic acid levels. This leads to a high GABA content, expressed per gram of food product in fried potatoes (165 µg/g FW in fried potato versus 103 µg/g FW in raw potato, supplementary material 5). On the other hand, for glutamic acid, this concentrating effect of frying is not able to compensate for the frying-induced loss of glutamic acid (0.9 mg/g FW in fried potato versus 0.97 mg/g FW in raw potato, supplementary material 5).

It was chosen to investigate the effect of cooking on peeled potatoes, but they can also be prepared unpeeled. For potato consistently higher glutamic acid levels in tuber peel as compared to flesh was shown across multiple potato genotypes, while for GABA no consistent differences between peel and flesh were found. Talley et al. (1983) found that, in general, the potato peel was higher in nitrogen and free amino acid contents than the potato flesh, although the exception in their samples was for glutamic acid and GABA [41].

Conclusion

Tomato and potato genotypes relatively high in GABA, could be a good source of GABA as part of a natural GABA-rich diet. Other food sources may contain more GABA but are rarely eaten in larger quantities than potato and tomato. Melons for example can contain even up to 3000 µg/g FW GABA, but the average intake of melons is low in the EU (ranging from 2.3 gram per day in the Netherlands to 14 grams a day in Italy) [4,42,43]. This means that such high GABA-containing melons only contribute about 7-40 mg to the daily GABA intake. Based on the EU-average intake of 233 gram potato and 92 gram tomato per day and assuming the average genotype GABA content of raw potato (321 µg/g FW) and tomato (242 µg/g FW), people in the EU currently receive 75 mg GABA/day from potato and 22 mg/day from tomato consumption, which translates into 97 mg GABA/day total excluding losses or gains from cooking [17,18]. If the highest GABA potato (cv. 'Riviera') and tomato (cv. 'Madara') cultivars would be consumed, this intake could increase to 173 mg per day from potato and 90 mg per day from tomato, i.e. 263 mg GABA/day in total. With a focused effort on breeding, growth conditions and food treatments this intake could likely be even higher. However, it is yet unclear what would be an effective GABA dose to exert significant health effects in humans, because the human intervention studies that were done so far did not have proper controls to relate the observed effects to GABA alone [44–46]. We recently showed that in humans GABA is readily taken up into the blood stream upon consuming pureed tomatoes [16]. In addition, the GABA content of both tomato and potato is reproducible across different years of harvest and seems to be quite

stable during further preparation like cooking. In conclusion, high GABA-containing potatoes and tomatoes could be promising additions to a healthy diet.

References

[1] B. Redruello, A. Szwengiel, V. Ladero, B. del Rio, M.A. Alvarez, Identification of technological/metabolic/environmental profiles of cheeses with high GABA contents, LWT. (2020) 109603. https://doi.org/10.1016/J.LWT.2020.109603.

[2] X. Yang, W. Hu, Z. Xiu, A. Jiang, X. Yang, G. Saren, Y. Ji, Y. Guan, K. Feng, Microbial Community Dynamics and Metabolome Changes During Spontaneous Fermentation of Northeast Sauerkraut From Different Households, Front Microbiol. 11 (2020) 1878. https://doi.org/10.3389/FMICB.2020.01878/BIBTEX.

[3] H. Herawati, D.N. Afifah, E. Kusumanigtyas, S. Usmiati, A.S. Soemantri, Miskiyah, E. Kamsiati, M. Bachtiar, Characterization of GABA (gamma-aminobutyric acid) levels some fermented food in Indonesia, IOP Conf Ser Earth Environ Sci. 819 (2021) 012068. https://doi.org/10.1088/1755-1315/819/1/012068.

[4] T. Toyoizumi, S. Ohba, K. suke Fujii, A. Ikegaya, H. Matsuura, T. Nakajima, Differential GABA concentration gradients are present in the edible parts of greenhouse melon (Cucumis melo L.) during all four seasonal croppings, Biosci Biotechnol Biochem. 83 (2019) 330–338. https://doi.org/10.1080/09168451.2018.1537774.

[5] T. Saito, C. Matsukura, M. Sugiyama, A. Watahiki, I. Ohshima, Y. Iijima, C. Konishi, T. Fujii, S. Inai, N. Fukuda, S. Nishimura, H. Ezura, Screening for γ-aminobutyric acid (GABA)-rich tomato varieties, Journal of the Japanese Society for Horticultural Science. 77 (2008) 242–250. https://doi.org/10.2503/jjshs1.77.242.

[6] K. Nakamura, K. Nara, T. Noguchi, T. Oshiro, H. Koga, Contents of gamma-aminobutyric acid (GABA) in potatoes and processed potato products, Journal of the Japanese Society for Food Science and Technology. (2006).

[7] M. Toyota, D. Spencer, S. Sawai-Toyota, W. Jiaqi, T. Zhang, A.J. Koo, G.A. Howe, S. Gilroy, Glutamate triggers long-distance, calcium-based plant defense signaling, 2018.

[8] B.G. Forde, P.J. Lea, Glutamate in plants: Metabolism, regulation, and signalling, in: J Exp Bot, Oxford Academic, 2007: pp. 2339–2358. https://doi.org/10.1093/jxb/erm121.

[9] S.A. Ramesh, S.D. Tyerman, M. Gilliham, B. Xu, γ-Aminobutyric acid (GABA) signalling in plants, Cellular and Molecular Life Sciences. 74 (2017) 1577–1603. https://doi.org/10.1007/s00018-016-2415-7.

[10] A.W. Bown, B.J. Shelp, Plant GABA: Not Just a Metabolite, Trends Plant Sci. 21 (2016) 811–813. https://doi.org/10.1016/j.tplants.2016.08.001.

[11] S.S. Scholz, M. Reichelt, D.W. Mekonnen, F. Ludewig, A. Mithöfer, Insect herbivory-elicited gaba accumulation in plants is a wound-induced, direct, systemic, and jasmonate-independent defense response, Front Plant Sci. 6 (2015) 1128. https://doi.org/10.3389/FPLS.2015.01128/BIBTEX.

[12] M. Takayama, H. Ezura, How and why does tomato accumulate a large amount of GABA in the fruit?, Front Plant Sci. 6 (2015) 612. https://doi.org/10.3389/fpls.2015.00612.

[13] J. Tian, H.N. Dang, J. Yong, W.-S. Chui, M.P.G. Dizon, C.K.Y. Yaw, D.L. Kaufman, Oral Treatment with γ-Aminobutyric Acid Improves Glucose Tolerance and Insulin Sensitivity by Inhibiting Inflammation in High Fat Diet-Fed Mice, PLoS One. 6 (2011) e25338. https://doi.org/10.1371/journal.pone.0025338.

[14] K. Hayakawa, M. Kimura, K. Kasaha, K. Matsumoto, H. Sansawa, Y. Yamori, Effect of a γ-aminobutyric acid-enriched dairy product on the blood pressure of spontaneously hypertensive and normotensive Wistar–Kyoto rats, British Journal of Nutrition. 92 (2004) 411. https://doi.org/10.1079/BJN20041221.

[15] J. Li, Z. Zhang, X. Liu, Y. Wang, F. Mao, J. Mao, X. Lu, D. Jiang, Y. Wan, J.-Y. Lv, G. Cao, J. Zhang, N. Zhao, M. Atkinson, D.L. Greiner, G.J. Prud'homme, Z. Jiao, Y. Li, Q. Wang, Study of GABA in Healthy Volunteers: Pharmacokinetics and Pharmacodynamics, Front Pharmacol. 6 (2015) 260. https://doi.org/10.3389/fphar.2015.00260.

[16] T.H. de Bie, M.G.J. Balvers, R.C.H. de Vos, R.F. Witkamp, M.A. Jongsma, The influence of a tomato food matrix on the bioavailability and plasma kinetics of oral gamma-aminobutyric acid (GABA) and its precursor glutamate in healthy men, Food Funct. (2022). https://doi.org/10.1039/D2FO01358D.

[17] Directorate-General for Agriculture and Rural Development, EU AGRICULTURAL OUTLOOK FOR THE AGRICULTURAL MARKETS AND INCOME 2021-2031, 2021. https://doi.org/10.2762/753688.

[18] R. Wijesinha-Bettoni, B. Mouillé, The Contribution of Potatoes to Global Food Security, Nutrition and Healthy Diets, American Journal of Potato Research 2019 96:2. 96 (2019) 139–149. https://doi.org/10.1007/S12230-018-09697-1.

[19] G.R. Skurray, N. Pucar, l-glutamic acid content of fresh and processed foods, Food Chem. 27 (1988) 177–180. https://doi.org/10.1016/0308-8146(88)90060-X.

[20] A. Fait, H. Fromm, D. Walter, G. Galili, A.R. Fernie, Highway or byway: the metabolic role of the GABA shunt in plants, Trends Plant Sci. 13 (2008) 14–19. https://doi.org/10.1016/J.TPLANTS.2007.10.005.

[21] A. Sorrequieta, G. Ferraro, S.B. Boggio, E.M. Valle, Free amino acid production during tomato fruit ripening: a focus on l-glutamate, Amino Acids. 38 (2010) 1523–1532. https://doi.org/10.1007/s00726-009-0373-1.

[22] J.T. Brosnan, M.E. Brosnan, Glutamate: a truly functional amino acid, Amino Acids. 45 (2013) 413–418. https://doi.org/10.1007/s00726-012-1280-4.

[23] S. Nonaka, C. Arai, M. Takayama, C. Matsukura, H. Ezura, Efficient increase of Γ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis, Sci Rep. 7 (2017) 1–14. https://doi.org/10.1038/s41598-017-06400-y.

[24] K. Zushi, N. Matsuzoe, Salt stress-enhanced γ-aminobutyric acid (GABA) in tomato fruit, in: Acta Hortic, International Society for Horticultural Science, 2007: pp. 431–435. https://doi.org/10.17660/ActaHortic.2007.761.59.

[25] P. Gramazio, M. Takayama, H. Ezura, Challenges and Prospects of New Plant Breeding Techniques for GABA Improvement in Crops: Tomato as an Example, Front Plant Sci. 11 (2020) 1. https://doi.org/10.3389/fpls.2020.577980.

[26] G. Pratta, R. Zorzoli, S.B. Boggio, L.A. Picardi, E.M. Valle, Glutamine and glutamate levels and related metabolizing enzymes in tomato fruits with different shelf-life, Sci Hortic. 100 (2004) 341–347. https://doi.org/10.1016/j.scienta.2003.08.004.

[27] G. Tommonaro, C.F. Morelli, M. Rabuffetti, B. Nicolaus, R. De Prisco, C. Iodice, G. Speranza, Determination of flavor-potentiating compounds in different Italian tomato varieties, J Food Biochem. 45 (2021) 13736. https://doi.org/10.1111/jfbc.13736.

[28] S. Baldina, M.E. Picarella, A.D. Troise, A. Pucci, V. Ruggieri, R. Ferracane, A. Barone, V. Fogliano, A. Mazzucato, Metabolite profiling of Italian tomato landraces with different fruit types, Front Plant Sci. 7 (2016) 664. https://doi.org/10.3389/fpls.2016.00664.

[29] A. Pęksa, J. Miedzianka, A. Nemś, E. Rytel, The free-amino-acid content in six potatoes cultivars through storage, Molecules. 26 (2021) 1322. https://doi.org/10.3390/molecules26051322.

[30] N.G. Halford, N. Muttucumaru, S.J. Powers, P.N. Gillatt, L. Hartley, J.S. Elmore, D.S. Mottram, Concentrations of free amino acids and sugars in nine potato varieties: Effects of storage and relationship with acrylamide formation, J Agric Food Chem. 60 (2012) 12044–12055. https://doi.org/10.1021/jf3037566.

[31] C. Uri, Z. Juhász, Z. Polgár, Z. Bánfalvi, A GC-MS-based metabolomics study on the tubers of commercial potato cultivars upon storage, Food Chem. 159 (2014) 287–292. https://doi.org/10.1016/j.foodchem.2014.03.010.

[32] G. Dobson, T. Shepherd, S.R. Verrall, S. Conner, J.W. Mcnicol, G. Ramsay, L.V.T. Shepherd, H.V.D. Vies, D. Stewart, Phytochemical diversity in tubers of potato cultivars and landraces using a gc-ms metabolomics approach, J Agric Food Chem. 56 (2008) 10280–10291. https://doi.org/10.1021/jf801370b.

[33] N. Carreno-Quintero, A. Acharjee, C. Maliepaard, C.W.B. Bachem, R. Mumm, H. Bouwmeester, R.G.F. Visser, J.J.B. Keurentjes, Untargeted metabolic quantitative trait loci analyses reveal a relationship between primary metabolism and potato tuber quality, Plant Physiol. 158 (2012) 1306–1318. https://doi.org/10.1104/pp.111.188441.

[34] Animal Feed—General, Official Methods of Analysis of AOAC INTERNATIONAL. (2023). https://doi.org/10.1093/9780197610145.003.037.

[35] T. Akihiro, S. Koike, R. Tani, T. Tominaga, S. Watanabe, Y. Iijima, K. Aoki, D. Shibata, H. Ashihara, C. Matsukura, K. Akama, T. Fujimura, H. Ezura, Biochemical mechanism on GABA accumulation during fruit development in tomato, Plant Cell Physiol. 49 (2008) 1378–1389. https://doi.org/10.1093/pcp/pcn113.

[36] S. Koike, C. Matsukura, M. Takayama, E. Asamizu, H. Ezura, Suppression of γ-Aminobutyric Acid (GABA) Transaminases Induces Prominent GABA Accumulation, Dwarfism and Infertility in the Tomato (Solanum lycopersicum L.), Plant Cell Physiol. 54 (2013) 793–807. https://doi.org/10.1093/PCP/PCT035.

[37] A.M. Kinnersley, F.J. Turano, Gamma Aminobutyric Acid (GABA) and Plant Responses to Stress, Https://Doi.Org/10.1080/07352680091139277. 19 (2010) 479–509. https://doi.org/10.1080/07352680091139277.

[38] J.L. Li, Z.C. Tu, X.M. Sha, L. Zhang, D.R. Lin, K. Zeng, H. Wang, J.J. Pang, P.P. Tang, Effect of Frying on Fatty Acid Profile, Free Amino Acids and Volatile Compounds of Grass Carp (Ctenopharyngodon idellus) Fillets, J Food Process Preserv. 41 (2017) e13088. https://doi.org/10.1111/JFPP.13088.

[39] Y. Ma, A. Wang, M. Yang, S. Wang, L. Wang, S. Zhou, C. Blecker, Influences of cooking and storage on γ-aminobutyric acid (GABA) content and distribution in mung bean and its noodle products, LWT. 154 (2022) 112783. https://doi.org/10.1016/J.LWT.2021.112783.

[40] Y. Takahashi, T. Sasanuma, T. Abe, Accumulation of gamma-aminobutyrate (GABA) caused by heat-drying and expression of related genes in immature

vegetable soybean (edamame), Breed Sci. 63 (2013) 205–210. https://doi.org/10.1270/JSBBS.63.205.

[41] E.A. Talley, R.B. Toma, P.H. Orr, Composition of Raw and Cooked Potato Peel and Flesh: Amino Acid Content, J Food Sci. 48 (1983) 1360–1361. https://doi.org/10.1111/J.1365-2621.1983.TB09234.X.

[42] C. van Rossum, E. Buurma-Rethans, C. Dinnissen, M. Beukers, H. Brants, M. Ocké, The diet of the Dutch : Results of the Dutch National Food Consumption Survey 2012-2016, (2020). https://doi.org/10.21945/RIVM-2020-0083.

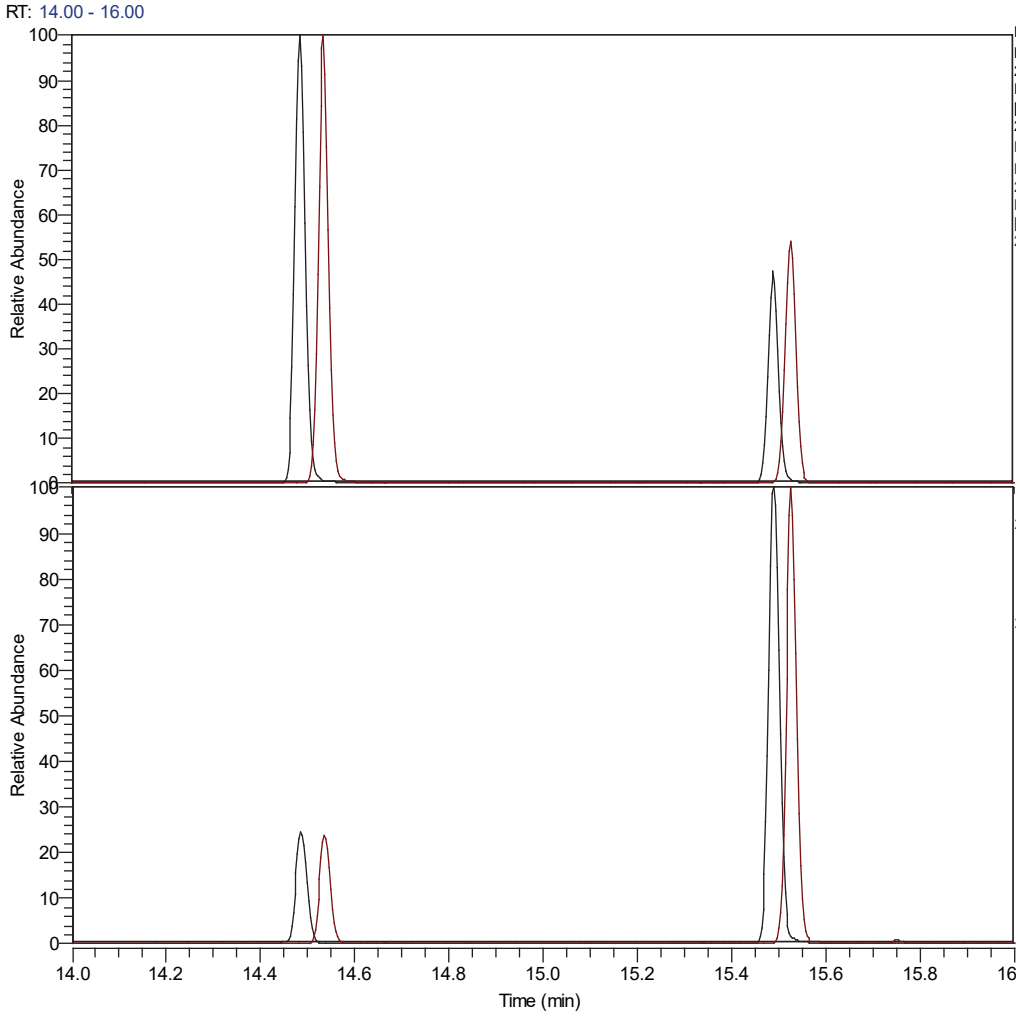
[43] C. Mertena, P. Ferrari, M. Bakker, A. Boss, A. Hearty, C. Leclercq, O. Lindtner, C. Tlustos, P. Verger, J.L. Volatier, D. Arcella, Methodological characteristics of the national dietary surveys carried out in the European Union as included in the European Food Safety Authority (EFSA) Comprehensive European Food Consumption Database, Http://Dx.Doi.Org/10.1080/19440049.2011.576440. 28 (2011) 975–995. https://doi.org/10.1080/19440049.2011.576440.

[44] K. Inoue, T. Shirai, H. Ochiai, M. Kasao, K. Hayakawa, M. Kimura, H. Sansawa, Blood-pressure-lowering effect of a novel fermented milk containing γ-aminobutyric acid (GABA) in mild hypertensives, Eur J Clin Nutr. 57 (2003) 490–495. https://doi.org/10.1038/sj.ejcn.1601555.

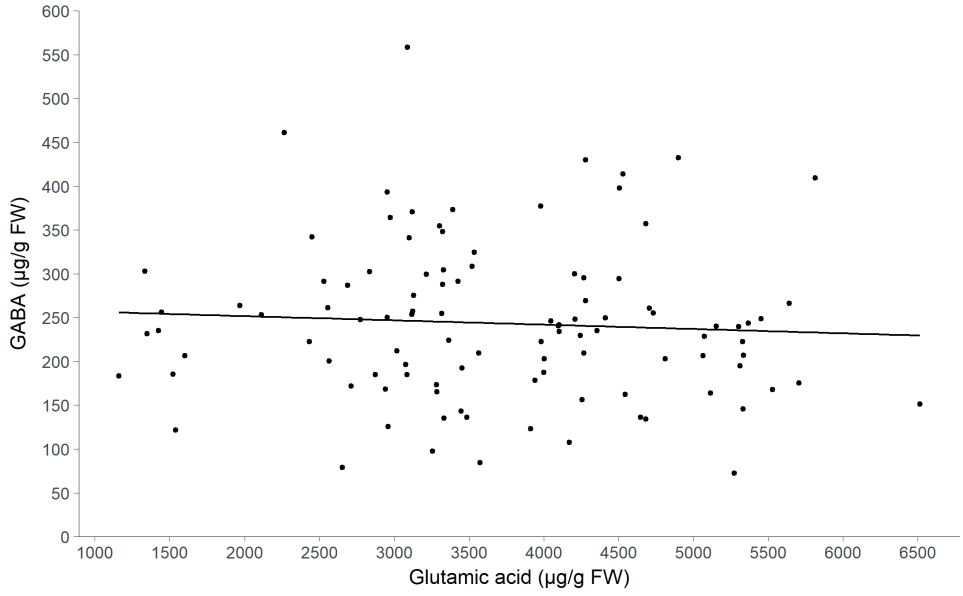
[45] H. Tanaka, K. Watanabe, M. Ma, M. Hirayama, T. Kobayashi, H. Oyama, Y. Sakaguchi, M. Kanda, M. Kodama, Y. Aizawa, The Effects of gamma-Aminobutyric Acid, Vinegar, and Dried Bonito on Blood Pressure in Normotensive and Mildly or Moderately Hypertensive Volunteers., J Clin Biochem Nutr. 45 (2009) 93–100. https://doi.org/10.3164/jcbn.09-04.

[46] M. Shimada, T. Hasegawa, C. Nishimura, H. Kan, T. Kanno, T. Nakamura, T. Matsubayashi, Anti-hypertensive effect of gamma-aminobutyric acid (GABA)-rich Chlorella on high-normal blood pressure and borderline hypertension in placebo-controlled double blind study, Clin Exp Hypertens. 31 (2009) 342–354. https://doi.org/10.1080/10641960902977908.

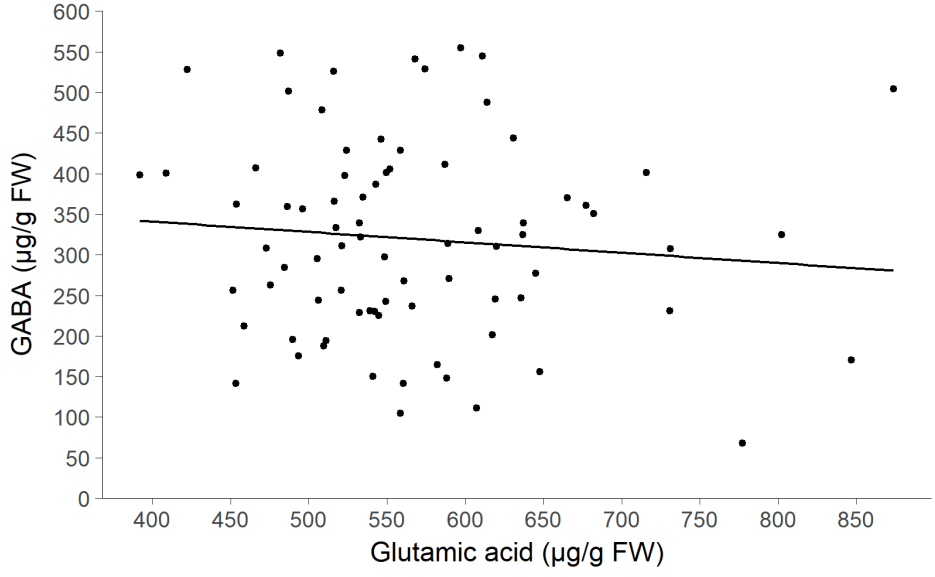
Supplementary material



Supplementary material 1: Typical GCMS chromatogram of standards (upper trace) and potato tuber extract (lower trace). Indicated values are the retention times and m/z values of d6-GABA, GABA, d5-glutamate and glutamate, respectively



Supplementary material 2: scatter plot, plotting the GABA content per gram of fresh weight against the glutamate content per gram of fresh weight in all measured tomato genotypes



Supplementary material 3: scatter plot, plotting the GABA content per gram of fresh weight against the glutamate content per gram of fresh weight in all measured potato genotypes

Supplementary material 4: Dry weight (%) of different prepared potatoes and tomatoes (n=3). Data reported as mean ± SD.

	Preparation	Dry weight (%)
Potato	Raw	16.6 ± 0.2
	Boiled	19.0 ± 1.4
	Fried	31.9 ± 2.1
	Steamed	18.6 ± 1.0
	Baked	22.4 ± 1.2
	Pan fried	23.3 ± 5.3
	Microwaved	21.1 ± 1.5
Tomato	Raw	4.6 ± 0.23
	Boiled	4.9 ± 0.26
	Baked	6.2 ± 0.30

Supplemental material 5: GABA and glutamic acid content (in µg/g FW and mg/g FW respectively) of different prepared potatoes and tomatoes (n=3). Data reported as mean ± SD. Note: not corrected for water losses or gains.

	Preparation	GABA (µg/g)	Glutamic acid (mg/g)
Potato	Raw	103.71 ± 7.95	0.97 ± 0.02
	Boiled	87.20 ± 15.40	0.98 ± 0.01
	Fried	165.03 ± 9.47	0.90 ± 0.10
	Steamed	94.22 ± 5.69	0.96 ± 0.02
	Baked	138.78 ± 17.11	0.95 ± 0.05
	Pan fried	116.34 ± 7.79	0.94 ± 0.02
	Microwaved	107.29 ± 7.26	0.95 ± 0.03
Tomato	Raw	211.74 ± 49.18	1.80 ± 0.23
	Boiled	314.92 ± 43.11	1.24 ± 0.23
	Baked	278.98 ± 38.29	1.27 ± 0.56

Chapter 3

Development and validation of a UPLC-MS/MS method for the simultaneous determination of gamma-aminobutyric acid and glutamic acid in human plasma

Authors: Tessa H. de Bie[a, b], Renger F. Witkamp[a], Maarten A. Jongsma[b], Michiel G.J. Balvers[a]

Affiliations:

[a] Division of Human Nutrition and Health, Wageningen University & Research, P.O. Box 17 6700 AA Wageningen, The Netherlands

[b] Wageningen Plant Research, Wageningen University & Research, P.O. Box 16 6700 AA Wageningen, The Netherlands

Published in the Journal of Chromatography B 2021 Feb 1;1164:122519

Abstract

Gamma-aminobutyric acid (GABA) and its precursor glutamic acid are important neurotransmitters. Both are also present in peripheral tissues and the circulation, where abnormal plasma concentrations have been linked to specific mental disorders. In addition to endogenous synthesis, GABA and glutamic acid can be obtained from dietary sources. An increasing number of studies suggest beneficial cardio-metabolic effects of GABA intake, and therefore GABA is being marketed as a food supplement. The need for further research into their health effects merits accurate and sensitive methods to analyze GABA and glutamic acid in plasma. To this end, an ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method was developed and validated for the quantification of GABA and glutamic acid in human plasma. Samples were prepared by a protein precipitation step and subsequent solid phase extraction using acetonitrile. Chromatographic separation was achieved on an Acquity UPLC HSS reversed phase C18 column using gradient elution. Analytes were detected using electrospray ionization and selective reaction monitoring. Standard curve concentrations for GABA ranged from 3.4 to 2500 ng/mL and for glutamic acid from 30.9 ng/mL to 22500 ng/mL. Within- and between-day accuracy and precision were < 10% in quality control samples at low, medium and high concentrations for both GABA and glutamic acid. GABA and glutamic acid were found to be stable in plasma after freeze-thaw cycles and up to 12 months of storage. The validated method was applied to human plasma from 17 volunteers. The observed concentrations ranged between 11.5 and 20.0 ng/ml and 2269 and 7625 ng/ml for respectively GABA and glutamic acid. The reported method is well suited for the measurement of plasma GABA and glutamic acid in pre-clinical or clinical studies.

Keywords

UPLC-MS/MS; SPE; GABA; glutamic acid; plasma

1. Introduction

Gamma-amino butyric acid (GABA) and glutamic acid (fig. 1.) are non-essential amino acids and mainly known as inhibitory and excitatory neurotransmitters, respectively. Next to this, glutamic acid has a central position in amino acid metabolism and has a signalling function in for example the pancreas and the gut where it regulates postprandial responses after protein ingestion [1–3]. GABA can be synthesized from glutamic acid via glutamic acid decarboxylase. Next to its actions as a neurotransmitter, GABA plays a role as a signalling molecule in peripheral tissues. For example, GABA is involved in regulating hormone secretion in the pancreas and its receptors are found on immune cells [4–6]. In addition to their localization in tissues, GABA and glutamic acid are circulating in the blood stream [7,8]. In healthy volunteers, mean (±SD) GABA concentrations of 13.40 ± 2.75 ng/mL are reported [9]. Lower Plasma GABA concentrations have been associated with mental disorders like bipolar disorder, depression and schizophrenia [9–13], while autism has been associated with higher Plasma GABA concentrations [14,15]. Literature regarding normal plasma glutamic acid concentrations is inconsistent [16]. However, significantly higher plasma glutamic acid concentrations compared to control values have been reported for patients with depression and autism [16–19]. At the same time, lower glutamic acid concentrations are associated with schizophrenia [20].

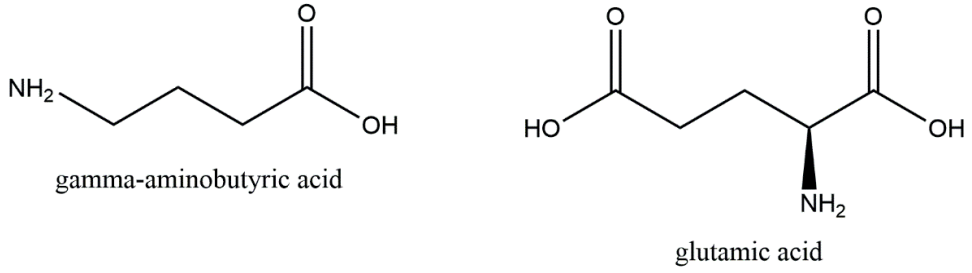


Fig. 1. Molecular structures of the amino acids GABA and glutamic acid.

GABA and glutamic acid are both available from dietary sources. GABA is only present as free amino acid, while glutamic acid is also one of the most abundant amino acids in dietary protein. Food products rich in GABA and glutamic acid include fermented foods and tomatoes [21,22]. In addition, monosodium glutamic acid is added as a flavouring agent to many food products. Oral administration of GABA and glutamic acid has acute effects on their circulating concentrations, presumably reflecting the plasma kinetics of uptake and distribution. Although 96% of dietary glutamic acid is instantly used as metabolic substrate by the intestine, a single oral dose of 150 mg/kg body weight has been reported to increase the plasma glutamic acid concentrations 10-fold over baseline within 50 minutes after ingestion [8,23]. GABA was also found to be rapidly absorbed in humans, producing a peak concentration 10 to 350 fold above baseline at 1 - 1,5 hours after ingestion of a dose of 2 grams [7]. Both amino acids are

most likely unable to pass the blood-brain barrier, so oral administration is unlikely to influence brain GABA and glutamic acid concentrations directly [24,25].

More recently, oral administration of GABA was shown to have beneficial effects in animal models of diabetes. GABA administration was able to induce β-cell replication and to reverse chemically induced diabetes [26–28]. The potential health effects of GABA in humans are currently under investigation (NCT04144439, NCT03635437, NCT03721991, NCT02002130, NCT04144439; ClinicalTrials.gov). To better comprehend the physiological effects of exogenously administered GABA, more insight in its plasma kinetics is needed. This requires sensitive methods that allow for the reliable quantification of GABA and its precursor glutamic acid in plasma. The development of chromatographic methods for small polar analytes is challenging. Many methods, using reversed phase or HILIC chromatography for example, are already available that measure these analytes in plant material, brain tissue, cerebrospinal fluid and urine [29–33].

Although some methods have been described that measure GABA in plasma, these have specific disadvantages, such as the need for derivatization or insufficient sensitivity to detect GABA in the bloodstream [34–36]. The present paper describes an ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) method that has been optimized to simultaneously measure GABA and glutamic acid in human plasma. This method is simple and robust and sufficiently sensitive, accurate and precise to quantify endogenous plasma concentrations of GABA and glutamic acid.

2. Materials and methods

2.1. Chemicals, materials and reagents

GABA, L-glutamic acid and internal standards gamma-aminobutyric acid-2,2-d2 (GABA-d2, 98% atom % D, 99% chemical purity) and glutamic acid-2,3,3,4,4-d5 (97 atom % D, 98% chemical purity) were purchased from Sigma–Aldrich (Munich, Germany). Acetonitrile (ACN), methanol and formic acid (ULC–MS grade) were purchased from Biosolve (Valkenswaard, the Netherlands). Milli-Q purified (MQ) water was used for all relevant preparations (ultrapure water system, arium 611UF, Sartorius Stedim Biotech GmbH, Göttingen, Germany). Blood samples were collected from apparently healthy volunteers in vacutainer plastic K2EDTA tubes purchased from Becton Dickinson (Etten-Leur, the Netherlands). Plasma (EDTA) was separated by centrifugation at 3000g for 10 minutes at 4 °C. A pooled plasma sample obtained by mixing plasma from 5 volunteers was snap-frozen, aliquoted and stored at -80 °C until further analysis.

2.2. Preparation of stock solutions, calibration standards and quality control samples

Stock solutions of GABA (10 mg/mL) and internal standard GABA-d2 (1 mg/mL), glutamic acid (1 mg/mL) and internal standard glutamic acid-d5 (1 mg/mL) were prepared in MQ water. Working solutions of 1 mg/mL GABA and glutamic acid were diluted to prepare a 7-point calibration curve in MQ water. The 7 calibration standards were prepared by 3-fold serial dilutions with a starting concentration of 2500 ng/mL for GABA and 22500 ng/mL for glutamic acid. Highest calibrator concentrations were based on the expected range in plasma concentrations after oral intake. All calibration standards contained 200 ng/mL of internal standard GABA-d2 and 2000 ng/mL of internal standard glutamic acid-d5.

Working solutions of 10 µg/mL, 1 µg/mL and 0.1 µg/mL for GABA and working solutions of 100 µg/ml, 10 µg/mL and 1 µg/mL of glutamic acid in MQ water were used for the preparation of the quality control (QC) samples. The QC samples were prepared by spiking GABA and glutamic acid into 100 µL human plasma from the human plasma pool. All stock solutions and calibration standards were stored at -20 °C. Pooled human plasma was stored at -80 °C in aliquots.

2.3. Sample preparation

To 100 µL of plasma, 400 µL of ACN containing 0.1% v/v formic acid (FA) was added. Internal standards were added to correspond to a concentration of 200 ng/mL GABA-d2 and 2000 ng/mL glutamic acid-d5 in the final extract. Samples were mixed at 1400 rpm (Eppendorf thermomixer, Eppendorf, Hamburg, Germany) for 5 minutes and centrifuged at 15000g for 15 minutes. The supernatant was diluted to 95% ACN with 1500 µL ACN. The extract was purified with solid phase extraction (SPE) using BondElut C8 200 mg SPE cartridges (Agilent, Santa Clara, United States). The samples were loaded onto cartridges, previously conditioned with 1 mL methanol and 1 mL 50% methanol in MQ water. Cartridges were washed with 2 mL 95% ACN and the analytes were eluted with 2 mL 80% ACN. Samples were evaporated to dryness in a vacuum concentrator (RVC 2-33 CDplus, Martin Christ, Osterode am Harz, Germany) for 3 hours at 30 °C and dissolved in 100µL MQ water with 0.1% v/v FA. The extracts were stored at -80 °C until analysis.

2.4. UPLC-MS/MS

Extracts were analysed for GABA and glutamic acid concentrations using an Acquity I-class UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp, Etten-leur, the Netherlands). Chromatographic separation was performed using an Acquity UPLC HSS reversed phase C18 column 2.1 x 150 mm with 1.8 µm particle size (Waters Corp, Milford, MA, United States). Column temperature was set to 30 °C, the sample temperature was set to 10 °C and the injection volume was 5 µL. Eluent A consisted of MQ water with 0.1% v/v FA, eluent B consisted of 100% methanol. The

following gradient was applied: 98% A; 0.0 – 3.0 min, 98% - 50% A; 3.0 – 6.0 min, 50% A; 6.0 – 8.0 min, 98% A; 8.0 – 10.0 min. Total runtime was 10 minutes and the eluent flowed at a rate of 0.15 mL/min. Electrospray ionization in positive mode (ESI-pos) was used for all analytes. Compounds were tuned individually to obtain optimal signals. The MS was run in selective reaction mode (SRM). Settings were used as follows: capillary voltage 3.05 kV, desolvation temperature 600 °C, source temperature 150 °C, cone gas flow 150 L/h, desolvation gas flow 1000 L/h and collision gas flow was 0.13 ml/min. The SRM settings are shown in Table 1. Data acquisition and quantification were performed using MassLynx version 4.1. Quantification was performed against a linear, 1/x weighted, regression curve based on the duplicate injection of calibration standards.

Table 1. SRM settings for GABA, glutamic acid, and internal standards.

Analyte	Precursor (*m/z*)	Product (*m/z*)	Collision energy
GABA	104.1	87.0	8
GABA-d2	106.1	89.1	10
Glutamic acid	148.0	130.0	10
Glutamic acid-d5	153.0	135.0	10

2.5. Method validation

2.5.1. Calibration, linearity, sensitivity and carry-over

Quantification was performed using a 7-point calibration curve (see section 2.2.) which was based on the duplicate analysis of calibrators using regression analysis with 1/x weighing. The peak area ratio of GABA/GABA-d2 and glutamic acid/glutamic acid-d5 were plotted against the analyte concentrations. The lower limit of quantification (LLOQ) was determined as the lowest concentration of the calibrator curve (n=3 curves) which did not deviate more than 20% from the actual concentration. The limit of detection (LOD) was assessed using a dilution series of GABA and glutamic acid in a range from 3.43 to 0.04 ng/mL for GABA and in a range from 277.8 to 3.43 ng/mL for glutamic acid. The LOD was defined as the concentration of analyte with a signal to noise ratio equal to 3. With linear regression (GraphPad Prism 5) the concentrations of GABA and glutamic acid with a S/N ratio of 3 were determined. Carry-over was determined by injecting a blank containing MQ water and 0.1% v/v FA immediately after the highest calibration standard. Carry-over was found acceptable if the peak area was ≤20% of the LLOQ.

2.5.2. Matrix effects and recovery

Since GABA and glutamic acid are endogenously present in human plasma, deuterated internal standards were spiked before and after extraction to establish matrix effects and recovery. To assess recovery, human plasma samples were spiked prior to sample

extraction (pre-spiked) with 200 ng/mL GABA-d2 and 2000 ng/mL glutamic acid-d5 or were spiked with the internal standards only after extraction (post-spiked). For each condition 3 replicates were prepared. The average internal standard peak area of the pre-spiked samples was expressed as a percentage of the post-spiked samples to determine the percentage recovery. To determine the matrix effect, the average peak area of the post-spiked plasma samples was compared to direct analysis of the internal standard solutions in clean solvent with the same concentration. Both the internal standard solution and the post-spiked plasma samples were prepared with solid phase extraction as is described in section 2.3.

2.5.3. Accuracy and precision

The within- and between-day accuracy and precision of the method were determined with low, medium and high QC concentrations spiked to a pooled human plasma sample. No GABA/glutamic acid free plasma is available since they are endogenously present in plasma. On top of the endogenously present GABA, plasma was spiked with 15 ng/mL (low), 150 ng/mL (medium), and 1500 ng/mL (high) GABA. These concentrations were chosen based on a previous report which showed that mean plasma GABA concentrations may range between ~10 up to ~ 1.000 ng/mL after the administration of GABA supplements, with outliers at > 5.000 ng/mL [7]. For glutamic acid, plasma was spiked with 1000 ng/mL (low), 5000 ng/mL (medium) and 15000 ng/mL (high). Five replicates of unspiked plasma and (spiked) QC samples were prepared, which was repeated on three days. Each analytical batch consisted of validation samples from one day and a duplicate calibration curve. Accuracy was calculated as [accuracy (Δ%) = ((measured concentration – endogenous concentration) * 100)/spiked concentration]. To determine precision, the relative standard deviation (RSD) was used. RSD was calculated as [RSD = (SD * 100)/mean]. Accuracy and precision were calculated both within- and between-day, to calculate the between-day accuracy and precision the average value of each day was taken.

2.5.4. Stability and re-injection reproducibility

Since GABA and/or glutamic acid are commonly determined in plasma, we determined pre-analytical stability only in plasma. The effects of one, two and three freeze-thaw cycles were assessed with three replicates of human plasma from a pool that was frozen at -80 °C. After each freeze-thaw cycle the samples were frozen again at -80 °C for 24 hours. Storage stability was assessed using the same human plasma pool. Three replicates were stored at -80 °C for either 1 month, 3 months, 6 months or 12 months. GABA and glutamic acid concentrations after freeze-thaw cycles and after storage were compared to plasma from the same plasma pool that was prepared immediately after blood drawing, without freezing and storage of the plasma sample. Prepared extracts were stored at -80 °C overnight for all conditions. The re-injection reproducibility of extracts was determined by re-injection after one week of storage at -80 °C. The GABA and glutamic acid concentrations of the re-injected extracts were calculated against both the original calibration curve as well as a freshly prepared

calibration curve. For stability analyses, a deviation of <15% from the original concentration is considered acceptable.

2.6. Measurement of the analytes in human plasma

For the measurement of GABA and glutamic acid, human blood samples were obtained from 17 volunteers by venepuncture. Sample collection was approved by the medical ethical committee of Wageningen University. Volunteers gave written informed consent before donation. Plasma (EDTA) was separated by centrifugation at 3000g for 10 minutes at 4 °C, snap-frozen and stored at -80 °C until further analysis.

3. Results and discussion

3.1. Method development and optimization

The initial method was based on previously published methods that measure GABA in plasma [7,34]. For our purpose, the sample preparation was optimised to reach sufficient sensitivity to detect endogenously present GABA. In our hands, a simple extraction step of protein precipitation with ACN, as described by others, did not result in acceptable performance [7,34]. Endogenously present GABA was not detected, due to an apparent lack of sensitivity.

Therefore, we explored the effectiveness of sample preconcentration and clean-up steps. Multiple SPE cartridges (such as Strata X-C and X-A cartridges) were tested for their suitability to achieve a near 100% recovery of GABA and glutamic acid (dissolved in MQ water) (data not shown). Among these, only BondElut C8 200 mg SPE cartridges were capable of retaining GABA. Elution with 20% ACN showed near 100% recovery of GABA as compared to a pure standard which did not go through the process of SPE. However, when a plasma sample containing GABA (endogenously present or spiked) was extracted with the same protocol, extraction was optimal when using 80% ACN during the elution step (Fig. 2). When eluting with 20% ACN during plasma extraction, barely any GABA is recovered. This shows that the matrix has a critical effect on the extraction behaviour of GABA. Various evaporation methods were compared, including vacuum concentration and evaporation under a gentle stream of nitrogen using the TurboVap system (Biotage, Uppsala, Sweden). Both methods displayed comparable performance (n = 5, 5.3% average difference in internal standard area) and are equally useful, with the TurboVap system allowing shorter drying times.

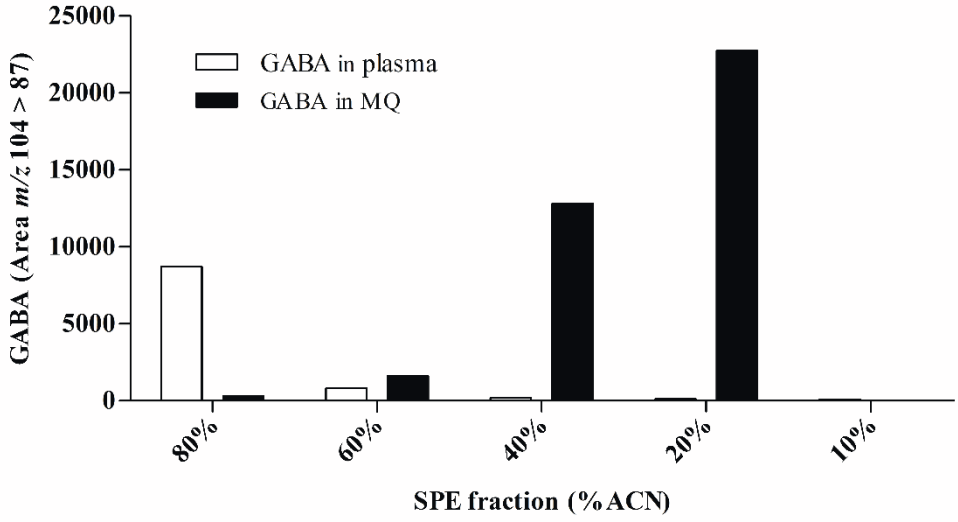


Fig. 2. Relative abundance of GABA in different SPE fractions. Plasma or MQ water was spiked with 200 ng/mL GABA. Samples were prepared according to section 2.3. During elution, decreasing percentages of ACN were used.

Next to the optimization of the sample preparation, the chromatographic separation was optimized. Reducing the UPLC flow rate to 150 µL/min increased the elution time and peak areas of GABA and glutamic acid. Chromatograms of GABA and glutamic acid at different flow rates are shown in fig. 3. By decreasing the flow rate from 0.3 mL/min to 0.15 ml/min the peak areas increased with 84% and 74% for GABA and glutamic acid respectively. Reversed-phase columns poorly retain polar analytes, this may lead to matrix effects and signal suppression. In method development we did not explore other chromatographic options. HILIC chromatography would be a valid option for these polar analytes and could increase retention time [32]. Derivatization of the analytes could also aid in increasing retention time and therefore reduce matrix effects [37]. While the current method is simple in use and has a robust quality, future research could focus on these methods if increased retention time would be called for.

In the method described, stability issues have required some attention. Previous reports have shown that glutamine can, under specific conditions, be converted to glutamic acid [38]. We have evaluated whether the glutamine present in plasma could lead to artefacts in our method by analyzing a pure glutamine standard solution. The glutamine sample showed a peak in the expected mass transition (147 > 130.1) for glutamine but showed no peak in the glutamic acid trace (Figure S1). It would therefore be unlikely that the glutamine present in plasma is a cause of artefacts in the glutamic acid analysis.

In addition, glutamic acid may undergo in-source conversion to pyroglutamic acid [39]. When injecting glutamic acid we did indeed detect the mass (130 > 84.1) that would be expected for pyroglutamic acid. A considerable amount of glutamic acid is converted in-source to pyroglutamic acid (pyroglutamic acid peak area is 1.4 times the glutamic acid peak area) (Figure S2). As investigated by Purwaha et al. (2014), this conversion to pyroglutamic acid is fully corrected for by the use of an appropriate internal standard, such as deuterium-labeled glutamic acid. Meaning that the [M+H] transition is suitable for glutamic acid quantification when glutamic acid-d5 is used as an internal standard [39].

Although internal standards with higher deuterium number are usually preferred, we chose GABA-d2 as an internal standard for GABA quantification. GABA-d6 was found unsuitable for the present application. Analyzed plasma samples that were not spiked with GABA-d6, contained peaks in the GABA-d6 trace (data not shown). We concluded that no significant conversion or contamination of GABA and GABA-d2 was detected in our analyses (Figure S3-S5).

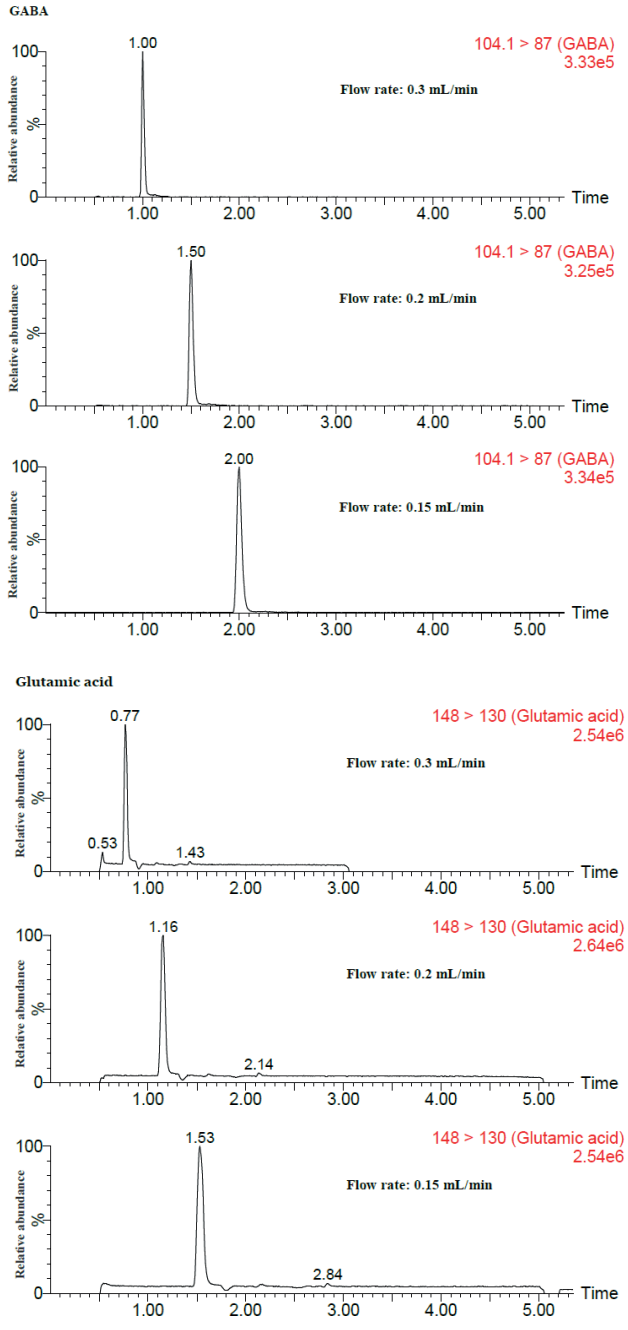


Fig. 3. SRM chromatograms of GABA (200 ng/mL) and glutamic acid (2000 ng/mL) obtained by UPLC-MS/MS at different UPLC flow rates.

3.2. Method validation and performance

3.2.1. Standard curve, sensitivity, linearity and carry-over

Calibration curves (n=3) were made with duplicate analysis of calibrators using regression analysis with 1/x weighing. The calibration curve for GABA ranged from 3.4 ng/mL to 2500 ng/mL, and for glutamic acid from 30.9 ng/mL to 22500 ng/mL. The r^2 values for the 3 obtained calibration curves were ≥0.997 for GABA and ≥0.998 for glutamic acid. The deviation of calculated concentrations was <20% from the actual concentration for all calibration points. At the LLOQ, accuracy was 6.7 Δ% for GABA and 2.0 Δ% for glutamic acid. Precision at the LLOQ was 10.5% (RSD) for GABA and 6.1% (RSD) for glutamic acid. Carry-over was assessed by injecting a blank after the highest calibrators; 2500 ng/mL GABA and 22500 ng/mL glutamic acid. Based on peak areas, carry-over was <7% of the LLOQ peak area. The sensitivity, matrix effects and recovery are shown in Table 2. At 0.12 ng/mL GABA can be detected with a signal to noise ratio of 3 (LOD). At 3.4 ng/mL GABA can reliably be quantified (LLOQ). The method is less sensitive for the detection of glutamic acid. The LOD of glutamic acid is 4.4 ng/mL at a signal to noise ratio of 3 and the LLOQ is at 30.9 ng/mL. This method is more sensitive than a comparable method by Busardo et. al, (2017) which was not able to measure endogenously present GABA [34]. Other available methods to measure GABA in plasma have a comparable or higher sensitivity [7,35,36]. The sensitivity of other available methods for the determination of glutamic acid are equally sensitive or slightly more sensitive than the current method [40–43]. With the achieved sensitivity this method is sufficiently sensitive to quantify endogenously present levels of GABA (~15 ng/mL) and glutamic acid (~4600 ng/mL) in human plasma (Fig. 4).

Table 2. Method sensitivity, matrix effects and recovery in human plasma.

	GABA	**Glutamic acid**
LOD[1] (ng/mL)	0.12	4.43
LLOQ[2] (ng/mL)	3.43	30.9
Matrix effect[3]	78.4%	70.9%
Recovery[4]	41%	22%

[1] The concentration with a S/N ratio of 3
[2] The lowest calibrator which deviates less than 20% from the actual concentration
[3] Percentage decrease in peak area in post-spiked samples as compared to the peak area of the spike solution
[4] Percentage of the pre-spiked samples peak area relative to the post-spiked samples peak area

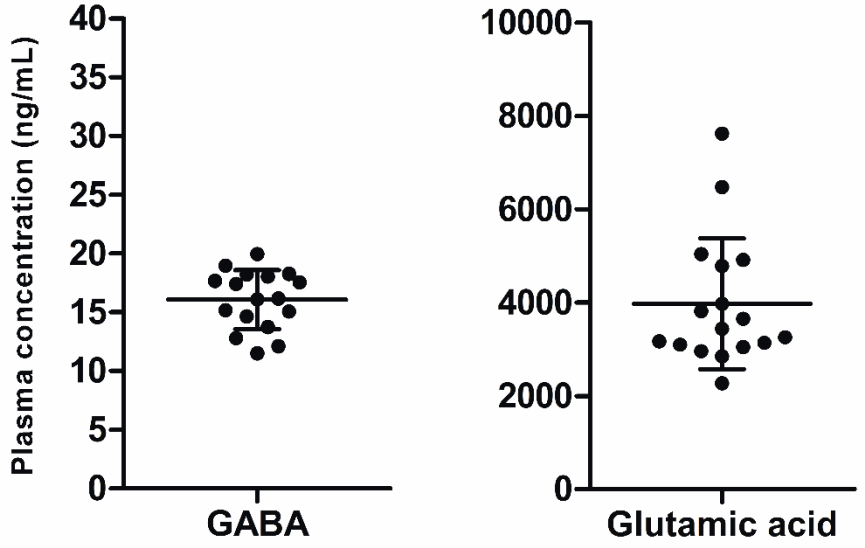


Fig. 4. Concentrations of GABA and glutamic acid in human plasma samples from 17 volunteers. Individual values as well as mean ± error bars representing SD are presented in the figure.

3.2.2. Matrix effects and recovery

Matrix effects were assessed by comparing the post-spiked plasma samples with the internal standard in clean solution. In the plasma samples relative to the internal standard solution, the peak area is decreased by 78.4% for GABA and for glutamic acid the peak area is decreased by 70.9% (Table 2). To determine the recovery, the peak area of the plasma samples that were pre-spiked with internal standard were compared with the post-spiked plasma samples. For GABA, 41% is recovered from the matrix while this is 22% for glutamic acid. In contrast, internal standard solution prepared similarly to the plasma samples has near 100% recovery as compared to a directly injected internal standard solution. This shows that the matrix has substantial influence on the recovery of the analytes. Future efforts may focus on improving recovery from plasma. Despite poor recovery and substantial matrix effects, the method is sufficiently sensitive as it is able to quantify endogenous levels of GABA and glutamic acid with sufficient sensitivity, accuracy and precision in human plasma (section 3.2.3).

3.2.3. Within- and between-day accuracy and precision

To determine the within- and between-day variation of the method, a pool was prepared from plasma of 5 volunteers. The precision and accuracy of the method were determined on 3 different days. On each day GABA and glutamic acid were spiked in five-fold into plasma at low, medium and high concentrations (see Table 3). Endogenous concentrations of GABA and glutamic acid were determined in the unspiked samples (average of 20 (SD 2,6) and 3864 (SD 188) ng/mL respectively), and these values were subtracted from the spiked samples to allow determination of accuracy. The mean concentration and the within- and between-day accuracy (Δ%) and precision (RSD (%)) are shown in Table 3. The method is capable of accurately and precisely quantifying GABA. Accuracy deviations ranged between 0.6% to 8.3% from the expected values, and precision ranged from 1.1% to 8.4%. The between-day precision and accuracy for GABA measurement is lower than 6.7% for all concentration levels. The within-day accuracy measurements of glutamic acid for all concentrations ranged from 0.2% to 5.1% from the nominal value and the precision ranged from 1.3% to 10%. The between-day deviation in accuracy and precision was lower than 3.5% for all concentration levels. It can be concluded that the method is accurate and precise for the determination of both GABA and glutamic acid in plasma.

Table 3. Within- and between-day accuracy and precision for GABA and Glutamic acid in quality control samples, presented concentrations are corrected for endogenous concentrations.

	GABA			**Glutamic acid**		
Spike (ng/mL)	**15**	**150**	**1500**	**1000**	**5000**	**15000**
Day 1						
Mean (SD), ng/mL[1]	16.0 (1.0)	140.8 (3.0)	1429.6 (15.7)	995.9 (99.7)	5256.3 (132.5)	15572.8 (974.1)
Δ%[2]	7.0	-6.1	-4.7	-0.4	5.1	3.8
RSD (%)[3]	6.2	2.2	1.1	10	2.5	6.3
Day 2						
Mean (SD), ng/mL	15.1 (1.3)	141.5 (2.6)	1435.6 (39.2)	1030.6 (37.4)	5159.4 (149.8)	15428.3 (348.6)
Δ%	0.6	-5.7	-4.3	3.1	3.2	2.9
RSD (%)	8.4	1.9	2.7	3.6	2.9	2.2
Day 3						
Mean (SD), ng/mL	14.3 (0.8)	137.6 (8.1)	1422.4 (33.2)	1047.4 (24.2)	4915.4 (235.2)	15033.5 (197.9)
Δ%	-4.5	-8.3	-5.2	4.7	-1.7	0.2
RSD (%)	5.4	5.9	2.3	2.3	4.8	1.3
Between-day						
Mean (SD), ng/mL	15.2 (0.9)	140.0 (2.1)	1429.2 (6.6)	1024.6 (26.3)	5110.4 (175.7)	15344.9 (279.2)
Δ%	1.0	-6.7	-4.7	2.5	2.2	2.3
RSD (%)	5.8	1.4	0.4	2.6	3.5	1.9

[1] The mean is the average concentration measured in the spiked samples subtracted by the mean endogenous concentration of the plasma pool

[2] Δ% is the percentage deviation of the measured concentration from the actual spiked value

[3] RSD (%) is the relative standard deviation, the standard deviation is expressed as a percentage of the mean

3.2.4. Stability and re-injection reproducibility

The freeze-thaw stability was assessed by comparing the analyte concentrations in plasma after up to three freeze-thaw cycles to plasma samples that were prepared immediately following venepuncture (n=3 for each condition). The concentrations of both analytes slightly increase after freeze-thaw cycles. The GABA concentration does not change more than 11.5% and the glutamic acid concentration does not change more than 3.3% as is shown in Table 4. Therefore, GABA and glutamic acid can be considered stable after multiple freeze thaw cycles. Long term storage was assessed by storing the plasma pool in aliquots for either 1 month, 3 months, 6 months or 12 months at -80 °C. Analyte concentrations were compared between the stored plasma samples and samples that were prepared immediately following venepuncture. As is shown in Table 4, GABA concentrations changed no more than 7.6% and glutamic acid concentrations changed no more than 6.9% during storage. Therefore, plasma can be stored for up to 12 months at -80 °C without any substantial concentration change of the analytes. However, it should be taken into account that plasma concentrations at higher than basal concentrations have not been tested for stability, and we therefore recommend to reduce storage time before sample preparation as much as possible when higher concentrations are expected.

Table 4. Stability of GABA and glutamic acid in human plasma.

	GABA			**Glutamic acid**		
	ng/mL	% of original concentration	RSD (%)	ng/mL	% of original concentration	RSD (%)
Freeze-thaw cycles						
No storage	21.6	100	2.0	4087.8	100	5.7
Freeze-thaw 1	24.1	111.5	6.0	4169.6	102.0	0.3
Freeze-thaw 2	23.5	108.6	6.5	4175.3	102.1	2.0
Freeze-thaw 3	24.0	110.8	6.9	4222.4	103.3	1.6
Long-term storage						
No storage	19.1	100	5.3	4340.7	100	6.4
1 month	18.5	96.7	4.3	4639.2	106.9	1.9
3 months	18.1	94.7	4.7	4348.4	100.2	0.9
6 months	19.2	100.4	2.4	4143.8	95.5	2.8
12 months	20.6	107.6	1.7	4295.1	99.0	0.4

Re-injection reproducibility was determined to assess the possibility of re-injecting the extracts in case of machine failure. QC samples and calibrators from the same batch were re-injected after 7 days of storage at -80 °C. Concentrations were calculated with the initial calibrator curve that was stored alongside the QC samples and a freshly prepared calibrator curve. The difference between the GABA and glutamic acid concentration calculated with the two different concentrations was negligible (<3%). Therefore, the initial calibrator does not have to be stored for re-injection. After re-injection, GABA concentrations changed no more than 6.9%, 8.2% and 2.8% for low, medium and high QC's respectively. Glutamic acid concentrations in low, medium and high QC's changed no more than 10.4%, 12.4% and 14.7% after re-injection. Thus, post-preparative storage for 7 days is considered acceptable since the results do not deviate more than 15% from the original analysis.

3.3. Application of the method to biological samples

The optimised method was applied to the measurement of GABA and glutamic acid in human plasma from 17 different apparently healthy volunteers. The values obtained are shown in fig. 4. An average (± SD) Plasma GABA concentration of 16.1 ± 2.5 ng/mL (range: 11.5 - 20.0 ng/mL) was found. The average plasma glutamic acid concentration was 3968 ± 1400 ng/mL (range: 2269 - 7625 ng/mL). Differences between individuals are larger for glutamic acid (RSD 35.3%) than for GABA (RSD 15.5%). Most individuals have glutamic acid concentrations between 2000 and 4000 ng/mL, a few individuals have higher concentrations of up to ~8000 ng/mL as is shown in fig. 4. Since glutamic acid is one of the most abundant amino acids in dietary protein, differences in diet between individuals could have introduced additional variation. In these individuals, plasma GABA and glutamic acid levels are not correlated (R^2 = 0.13). Representative chromatograms of GABA and glutamic acid and their respective deuterium labelled internal standards are shown in fig. 5.

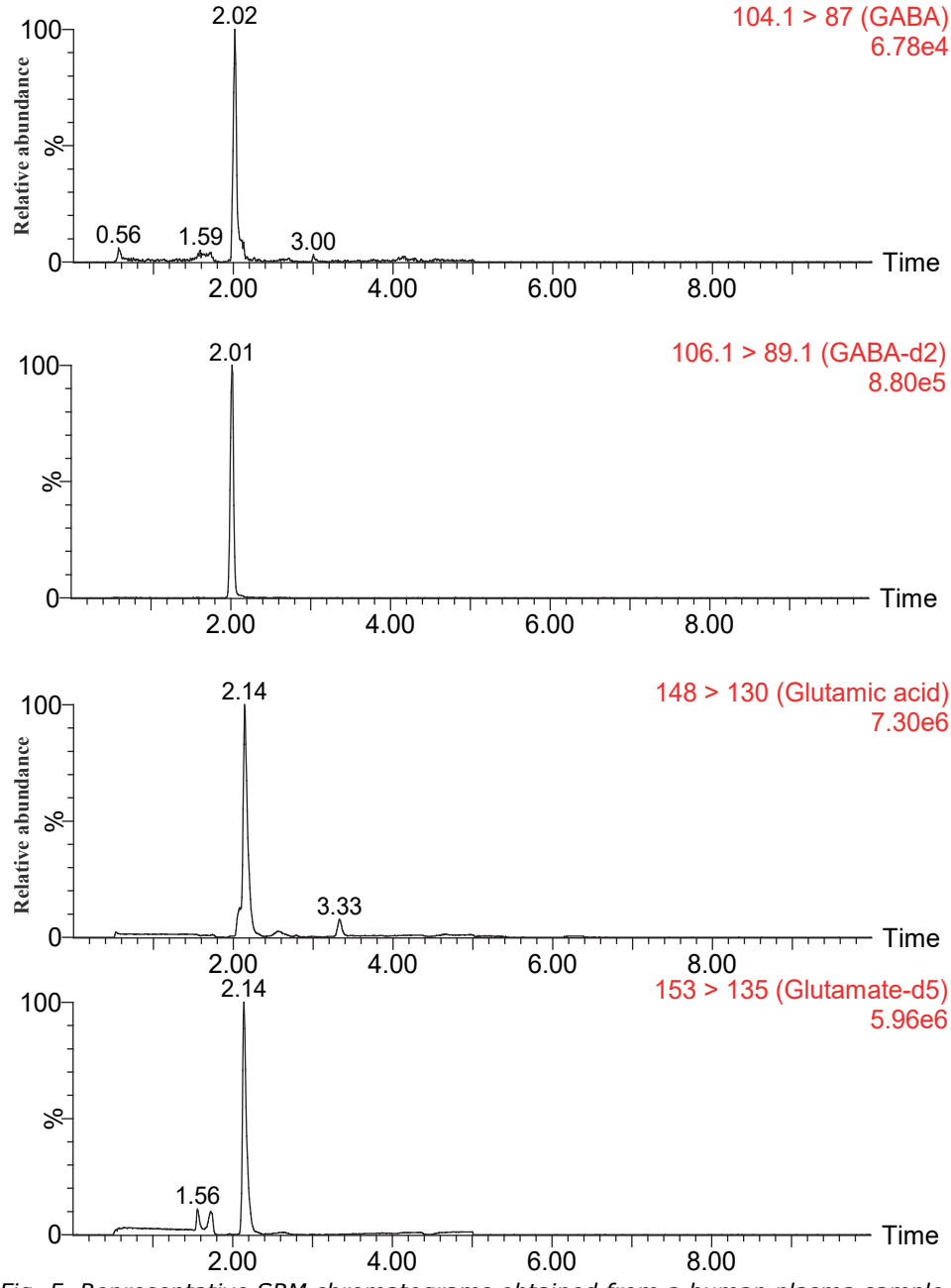


Fig. 5. Representative SRM chromatograms obtained from a human plasma sample of GABA and glutamic acid and their respective deuterium labelled internal standards.

The plasma glutamic acid concentrations are consistent with other validated methods [40,41,43]. The Plasma GABA concentrations are also consistent with values reported in literature [7,9,14,44]. However, it is good to note that two validated methods for the determination of GABA in plasma show substantially higher GABA levels in healthy volunteers [35,36]. Since there is no gold standard method available, only speculations are possible regarding the cause of these differences. As opposed to the current method several methods require a derivatization step, which increases retention of the analytes but on the other hand complicates sample preparation, reduces reliability or may create artefacts [37,45]. In addition, different calibrators could have been used. Song et. al, (2004) speculates that the often low recovery could explain the discrepancy between their results and others. However, our method corrects for losses in recovery by using internal standards. Future work should shed light on the causes of these discrepancies.

4. Conclusion

A UPLC-MS/MS method was successfully developed and validated for the simultaneous quantification of endogenous GABA and glutamic acid in human plasma. The analytical performance was found to be well within generally acceptable ranges. In addition, stability was for the first time thoroughly investigated. GABA and glutamic acid were found to be stable through multiple freeze-thaw cycles and long-term storage for up to 1 year. With the current method, we determined average GABA and glutamic acid concentrations of 16.1 ng/mL and 3968 ng/mL, respectively, which was in agreement with values reported by other groups. We conclude that the reported method is therefore well suited for the quantification of GABA and glutamic acid in human plasma.

References

[1] L. Cynober, Metabolism of Dietary Glutamate in Adults, Review Article Ann Nutr Metab. 73 (2018) 5–14. https://doi.org/10.1159/000494776.

[2] J. Du, X.-H. Li, Y.-J. Li, Glutamate in peripheral organs: Biology and pharmacology, Eur J Pharmacol. 784 (2016) 42–48. https://doi.org/10.1016/J.EJPHAR.2016.05.009.

[3] J.T. Brosnan, M.E. Brosnan, Glutamate: a truly functional amino acid, Amino Acids. 45 (2013) 413–418. https://doi.org/10.1007/s00726-012-1280-4.

[4] E. Adeghate, A.S. Ponery, GABA in the endocrine pancreas: cellular localization and function in normal and diabetic rats, Tissue Cell. 34 (2002) 1–6. https://doi.org/10.1054/TICE.2002.0217.

[5] A. Gladkevich, J. Korf, V.P. Hakobyan, K.V. Melkonyan, The peripheral GABAergic system as a target in endocrine disorders, Autonomic Neuroscience. 124 (2006) 1–8. https://doi.org/10.1016/J.AUTNEU.2005.11.002.

[6] A. Barragan, J.M. Weidner, Z. Jin, E.R. Korpi, B. Birnir, GABAergic signalling in the immune system, Acta Physiologica. 213 (2015) 819–827. https://doi.org/10.1111/apha.12467.

[7] J. Li, Z. Zhang, X. Liu, Y. Wang, F. Mao, J. Mao, X. Lu, D. Jiang, Y. Wan, J.-Y. Lv, G. Cao, J. Zhang, N. Zhao, M. Atkinson, D.L. Greiner, G.J. Prud'homme, Z. Jiao, Y. Li, Q. Wang, Study of GABA in Healthy Volunteers: Pharmacokinetics and Pharmacodynamics, Front Pharmacol. 6 (2015) 260. https://doi.org/10.3389/fphar.2015.00260.

[8] K.M. Di Sebastiano, K.E. Bell, T. Barnes, A. Weeraratne, T. Premji, M. Mourtzakis, Glutamate supplementation is associated with improved glucose metabolism following carbohydrate ingestion in healthy males, British Journal of Nutrition. 110 (2013) 2165–2172. https://doi.org/10.1017/S0007114513001633.

[9] A. Arrúe, R. Dávila, M. Zumárraga, N. Basterreche, M.A. González-Torres, B. Goienetxea, M.I. Zamalloa, J.B. Anguiano, J. Guimón, GABA and Homovanillic Acid in the Plasma of Schizophrenic and Bipolar I Patients, Neurochem Res. 35 (2010) 247–253. https://doi.org/10.1007/s11064-009-0048-z.

[10] H.-L. Cai, R.-H. Zhu, H.-D. Li, X.-H. Zhang, L. Hu, W. Yang, H.-S. Ye, Elevated plasma γ-aminobutyrate/glutamate ratio and responses to risperidone antipsychotic treatment in schizophrenia, Prog Neuropsychopharmacol Biol Psychiatry. 34 (2010) 1273–1278. https://doi.org/10.1016/J.PNPBP.2010.07.006.

[11] F. Petty, G.L. Kramer, M. Fulton, F.G. Moeller, A.J. Rush, Low Plasma GABA Is a Trait-Like Marker for Bipolar Illness, Neuropsychopharmacology. 9 (1993) 125–132. https://doi.org/10.1038/npp.1993.51.

[12] E. Küçükibrahimoğlu, M.Z. Saygın, M. Çalışkan, O.K. Kaplan, C. Ünsal, M.Z. Gören, The change in plasma GABA, glutamine and glutamate levels in fluoxetine- or S-citalopram-treated female patients with major depression, Eur J Clin Pharmacol. 65 (2009) 571–577. https://doi.org/10.1007/s00228-009-0650-7.

[13] F. Petty, G.L. Kramer, M. Fulton, L. Davis, A.J. Rush, Stability of plasma GABA at four-year follow-up in patients with primary unipolar depression, Biol Psychiatry. 37 (1995) 806–810. https://doi.org/10.1016/0006-3223(94)00226-S.

[14] D. Dhossche, H. Applegate, A. Abraham, P. Maertens, L. Bland, A. Bencsath, J. Martinez, Elevated plasma gamma-aminobutyric acid (GABA) levels in autistic youngsters: stimulus for a GABA hypothesis of autism., Med Sci Monit. 8 (2002) PR1-6. http://www.ncbi.nlm.nih.gov/pubmed/12165753 (accessed August 27, 2019).

[15] A.J. Russo, Correlation between Hepatocyte Growth Factor (HGF) and Gamma-Aminobutyric Acid (GABA) Plasma Levels in Autistic Children, Biomark Insights. 8 (2013) BMI.S11448. https://doi.org/10.4137/BMI.S11448.

[16] Z. Zheng, T. Zhu, Y. Qu, D. Mu, Blood Glutamate Levels in Autism Spectrum Disorder: A Systematic Review and Meta-Analysis, PLoS One. 11 (2016) e0158688. https://doi.org/10.1371/journal.pone.0158688.

[17] J.S. Kim, W. Schmid-Burgk, D. Claus, H.H. Kornhuber, Increased serum glutamate in depressed patients, Archiv For Psychiatrie Und Nervenkrankheiten. 232 (1982) 299–304. https://doi.org/10.1007/BF00345492.

[18] C. Shimmura, S. Suda, K.J. Tsuchiya, K. Hashimoto, K. Ohno, H. Matsuzaki, K. Iwata, K. Matsumoto, T. Wakuda, Y. Kameno, K. Suzuki, M. Tsujii, K. Nakamura, N. Takei, N. Mori, Alteration of Plasma Glutamate and Glutamine Levels in Children with High-Functioning Autism, PLoS One. 6 (2011) e25340. https://doi.org/10.1371/journal.pone.0025340.

[19] H. Mitani, Y. Shirayama, T. Yamada, K. Maeda, C.R. Ashby, R. Kawahara, Correlation between plasma levels of glutamate, alanine and serine with severity of depression, Prog Neuropsychopharmacol Biol Psychiatry. 30 (2006) 1155–1158. https://doi.org/10.1016/J.PNPBP.2006.03.036.

[20] A. Palomino, A. González-Pinto, A. Aldama, C. González-Gómez, F. Mosquera, G. González-García, C. Matute, Decreased levels of plasma glutamate in patients with first-episode schizophrenia and bipolar disorder, Schizophr Res. 95 (2007) 174–178. https://doi.org/10.1016/J.SCHRES.2007.06.012.

[21] M. Takayama, H. Ezura, How and why does tomato accumulate a large amount of GABA in the fruit?, Front Plant Sci. 6 (2015) 612. https://doi.org/10.3389/fpls.2015.00612.

[22] A. Sorrequieta, G. Ferraro, S.B. Boggio, E.M. Valle, Free amino acid production during tomato fruit ripening: a focus on l-glutamate, Amino Acids. 38 (2010) 1523–1532. https://doi.org/10.1007/s00726-009-0373-1.

[23] A. Battezzati, D.J. Brillon, D.E. Matthews, Oxidation of glutamic acid by the splanchnic bed in humans, n.d.

[24] E. Boonstra, R. de Kleijn, L.S. Colzato, A. Alkemade, B.U. Forstmann, S. Nieuwenhuis, Neurotransmitters as food supplements: the effects of GABA on brain and behavior, Front Psychol. 6 (2015) 1520. https://doi.org/10.3389/fpsyg.2015.01520.

[25] Q.R. Smith, Transport of Glutamate and Other Amino Acids at the Blood-Brain Barrier, J Nutr. 130 (2000) 1016S-1022S. https://doi.org/10.1093/jn/130.4.1016S.

[26] N. Soltani, H. Qiu, M. Aleksic, Y. Glinka, F. Zhao, R. Liu, Y. Li, N. Zhang, R. Chakrabarti, T. Ng, T. Jin, H. Zhang, W.-Y. Lu, Z.-P. Feng, G.J. Prud'homme, Q. Wang, GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes., Proc Natl Acad Sci U S A. 108 (2011) 11692–7. https://doi.org/10.1073/pnas.1102715108.

[27] J. Tian, H.N. Dang, J. Yong, W.-S. Chui, M.P.G. Dizon, C.K.Y. Yaw, D.L. Kaufman, Oral Treatment with γ-Aminobutyric Acid Improves Glucose Tolerance and Insulin Sensitivity by Inhibiting Inflammation in High Fat Diet-Fed Mice, PLoS One. 6 (2011) e25338. https://doi.org/10.1371/journal.pone.0025338.

[28] N. Ben-Othman, A. Vieira, M. Courtney, F. Record, E. Gjernes, F. Avolio, B. Hadzic, N. Druelle, T. Napolitano, S. Navarro-Sanz, S. Silvano, K. Al-Hasani, A. Pfeifer, S. Lacas-Gervais, G. Leuckx, L. Marroquí, J. Thévenet, O.D. Madsen, D.L. Eizirik, H. Heimberg, J. Kerr-Conte, F. Pattou, A. Mansouri, P. Collombat, Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis, Cell. 168 (2017) 73-85.e11. https://doi.org/10.1016/J.CELL.2016.11.002.

[29] J.A. Eckstein, G.M. Ammerman, J.M. Reveles, B.L. Ackermann, Analysis of glutamine, glutamate, pyroglutamate, and GABA in cerebrospinal fluid using ion pairing HPLC with positive electrospray LC/MS/MS, J Neurosci Methods. 171 (2008) 190–196. https://doi.org/10.1016/j.jneumeth.2008.02.019.

[30] K. Buck, P. Voehringer, B. Ferger, Rapid analysis of GABA and glutamate in microdialysis samples using high performance liquid chromatography and tandem mass spectrometry, J Neurosci Methods. 182 (2009) 78–84. https://doi.org/10.1016/j.jneumeth.2009.05.018.

[31] C. Defaix, A. Solgadi, T.H. Pham, A.M. Gardier, P. Chaminade, L. Tritschler, Rapid analysis of glutamate, glutamine and GABA in mice frontal cortex microdialysis samples using HPLC coupled to electrospray tandem mass spectrometry, J Pharm Biomed Anal. 152 (2018) 31–38. https://doi.org/10.1016/j.jpba.2018.01.039.

[32] K. Inoue, Y. Miyazaki, K. Unno, J.Z. Min, K. Todoroki, T. Toyo'oka, Stable isotope dilution HILIC-MS/MS method for accurate quantification of glutamic acid, glutamine, pyroglutamic acid, GABA and theanine in mouse brain tissues, Biomedical Chromatography. 30 (2016) 55–61. https://doi.org/10.1002/bmc.3502.

[33] J. Yan, U. Kuzhiumparambil, S. Bandodkar, N. Solowij, S. Fu, Development and validation of a simple, rapid and sensitive LC-MS/MS method for the measurement of urinary neurotransmitters and their metabolites, Anal Bioanal Chem. 409 (2017) 7191–7199. https://doi.org/10.1007/s00216-017-0681-3.

[34] F.P. Busardò, C. Kyriakou, E. Marchei, R. Pacifici, D.S. Pedersen, S. Pichini, Ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) for determination of GHB, precursors and metabolites in different specimens: Application to clinical and forensic cases, J Pharm Biomed Anal. 137 (2017) 123–131. https://doi.org/10.1016/J.JPBA.2017.01.022.

[35] Y. Song, M. Shenwu, D.M. Dhossche, Y.-M. Liu, A capillary liquid chromatographic/tandem mass spectrometric method for the quantification of γ-aminobutyric acid in human plasma and cerebrospinal fluid, Journal of Chromatography B. 814 (2005) 295–302. https://doi.org/10.1016/J.JCHROMB.2004.10.046.

[36] H.-L. Cai, R.-H. Zhu, H.-D. Li, Determination of dansylated monoamine and amino acid neurotransmitters and their metabolites in human plasma by liquid chromatography–electrospray ionization tandem mass spectrometry, Anal Biochem. 396 (2010) 103–111. https://doi.org/10.1016/J.AB.2009.09.015.

[37] P. Deng, Y. Zhan, X. Chen, D. Zhong, Derivatization methods for quantitative bioanalysis by LC-MS/MS, Bioanalysis. 4 (2012) 49–69. https://doi.org/10.4155/bio.11.298.

[38] C.B. AIRAUDO, A. GAYTE-SORBIER, P. ARMAND, Stability of Glutamine and Pyroglutamic Acid under Model System Conditions: Influence of Physical and Technological Factors, J Food Sci. 52 (1987) 1750–1752. https://doi.org/10.1111/j.1365-2621.1987.tb05926.x.

[39] P. Purwaha, L.P. Silva, D.H. Hawke, J.N. Weinstein, P.L. Lorenzi, An artifact in LC-MS/MS measurement of glutamine and glutamic acid: In-source cyclization to pyroglutamic acid, Anal Chem. 86 (2014) 5633–5637. https://doi.org/10.1021/ac501451v.

[40] Y.V. Tcherkas, A.D. Denisenko, Simultaneous determination of several amino acids, including homocysteine, cysteine and glutamic acid, in human plasma by isocratic reversed-phase high-performance liquid chromatography with fluorimetric detection, J Chromatogr A. 913 (2001) 309–313. https://doi.org/10.1016/S0021-9673(00)01201-2.

[41] M.P. Frank, R.W. Powers, Simple and rapid quantitative high-performance liquid chromatographic analysis of plasma amino acids, Journal of Chromatography B. 852 (2007) 646–649. https://doi.org/10.1016/J.JCHROMB.2007.01.002.

[42] H.C.M.T. Prinsen, B.G.M. Schiebergen-Bronkhorst, M.W. Roeleveld, J.J.M. Jans, M.G.M. de Sain-van der Velden, G. Visser, P.M. van Hasselt, N.M. Verhoeven-Duif, Rapid quantification of underivatized amino acids in plasma by hydrophilic

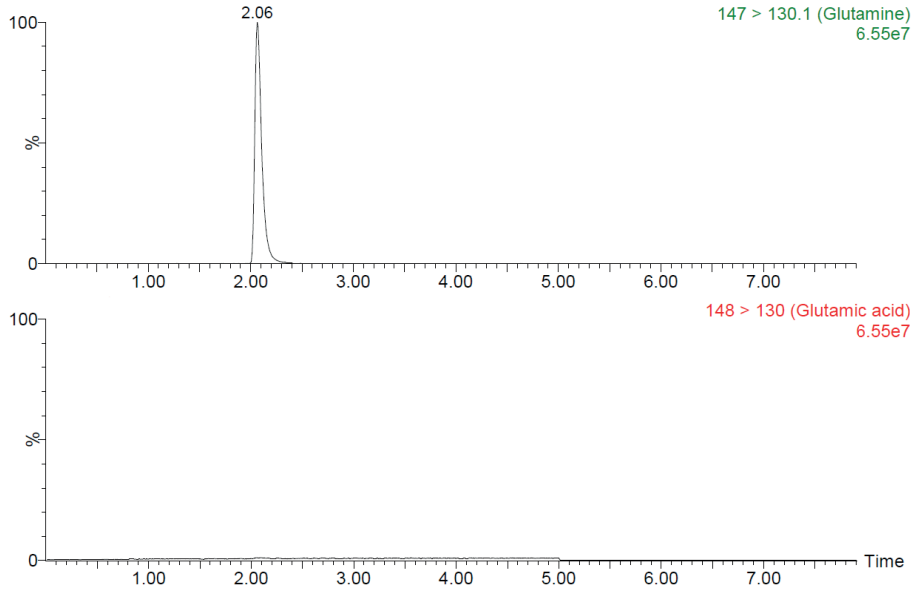
interaction liquid chromatography (HILIC) coupled with tandem mass-spectrometry, J Inherit Metab Dis. 39 (2016) 651–660. https://doi.org/10.1007/s10545-016-9935-z.

[43] D. Fekkes, A. van Dalen, M. Edelman, A. Voskuilen, Validation of the determination of amino acids in plasma by high-performance liquid chromatography using automated pre-column derivatization with o-phthaldialdehyde, J Chromatogr B Biomed Sci Appl. 669 (1995) 177–186. https://doi.org/10.1016/0378-4347(95)00111-U.

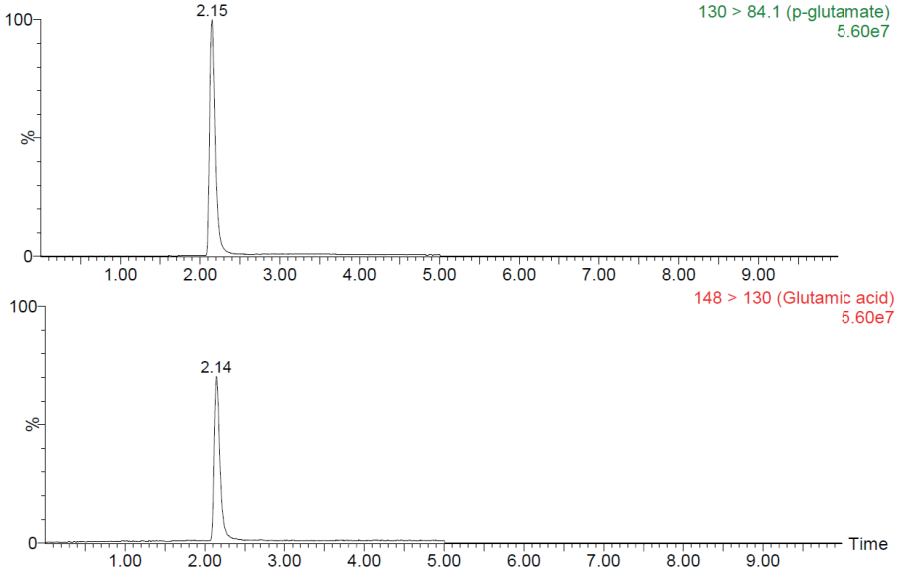
[44] F. Petty, G.L. Kramer, M. Fulton, L. Davis, A.J. Rush, Stability of plasma GABA at four-year follow-up in patients with primary unipolar depression, Biol Psychiatry. 37 (1995) 806–810. https://doi.org/10.1016/0006-3223(94)00226-S.

[45] H.H. Kanani, M.I. Klapa, Data correction strategy for metabolomics analysis using gas chromatography-mass spectrometry, Metab Eng. 9 (2007) 39–51. https://doi.org/10.1016/j.ymben.2006.08.001.

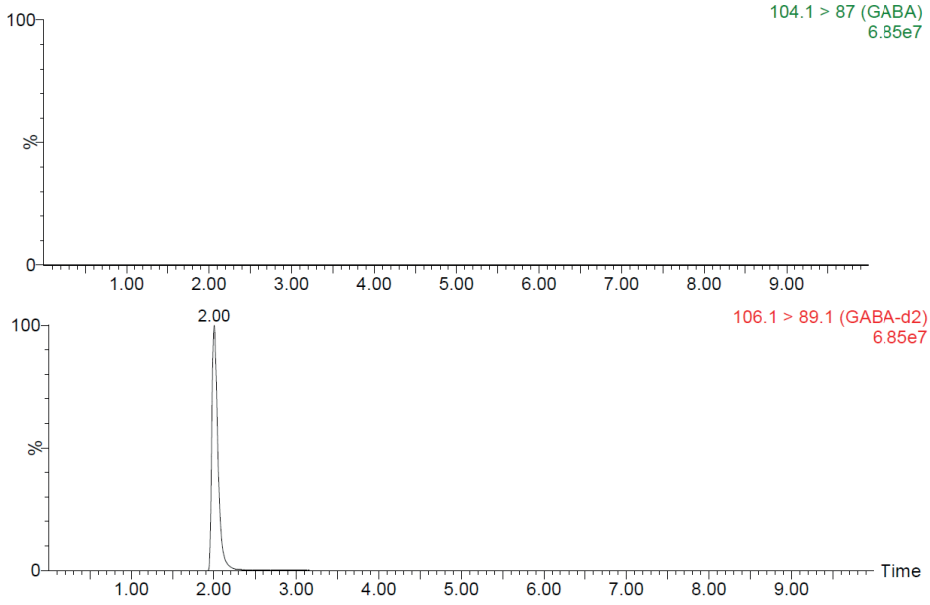
S1. SRM chromatograms of glutamine (10 ug/mL) showing the glutamic acid (lower) and glutamine (upper) trace



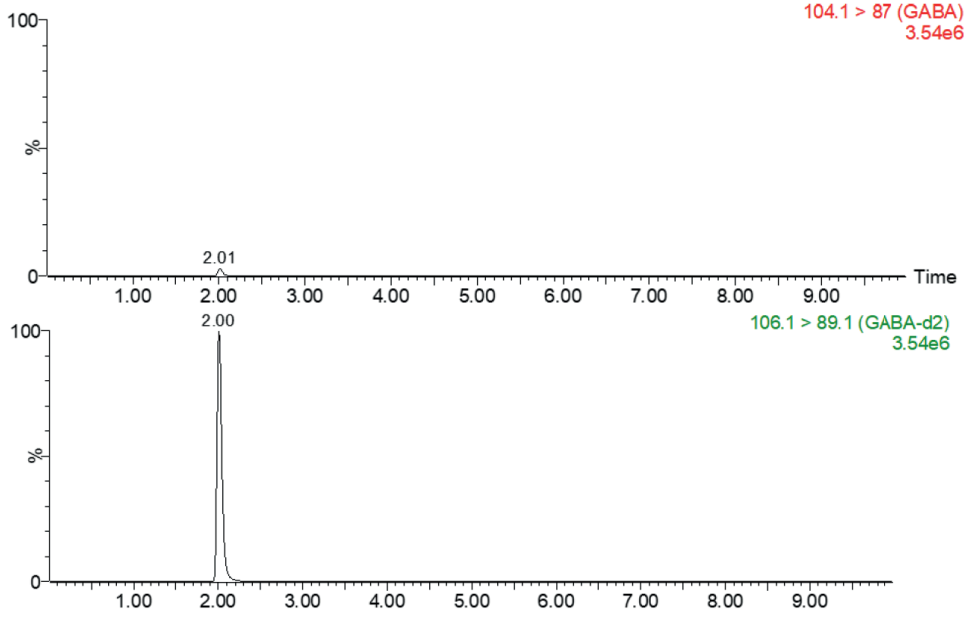
S2. SRM chromatograms of glutamic acid (10 ug/mL) showing the glutamic acid (lower) and pyroglutamic acid (upper) trace



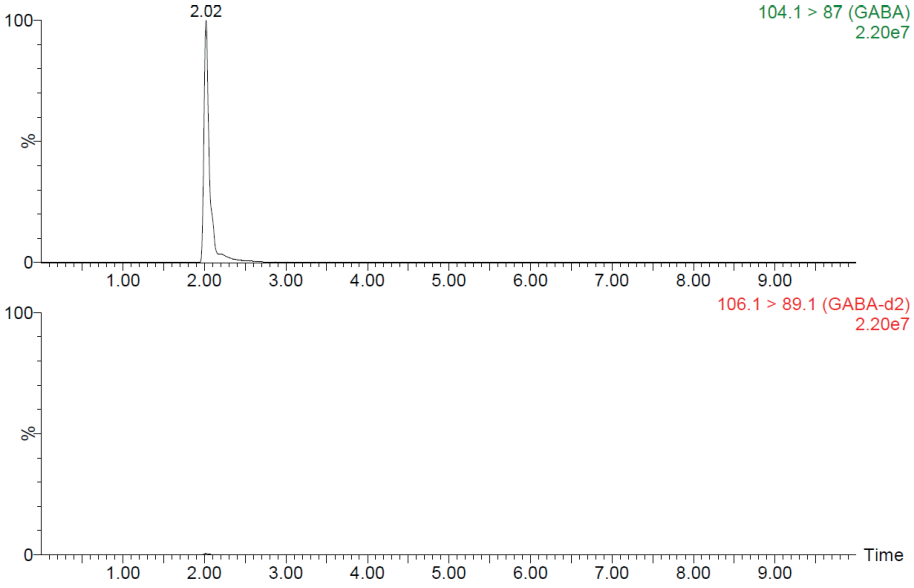
S3. SRM chromatograms of GABA-d2 standard (10 ug/mL) showing the GABA-d2 (lower) and GABA (upper) trace



S4. SRM chromatograms of lowest calibrator containing GABA (3.43 ng/mL) and GABA-d2 (200 ng/mL) showing the GABA-d2 (lower) and GABA (upper) trace



S5. SRM chromatograms of highest calibrator containing GABA (2500 ng/mL) and glutamic acid (22500 ng/mL) showing the GABA-d2 (lower) and GABA (upper) trace



Chapter 4

Relative bioavailability and plasma kinetics of oral gamma-aminobutyric acid (GABA) and its precursor glutamate from tomato in healthy men

Authors: Tessa H. de Bie[a, b], Michiel G.J. Balvers[a], Ric C.H. de Vos[b], Renger F. Witkamp[a], Maarten A. Jongsma[b]

Affiliations:

[a] Division of Human Nutrition and Health, Wageningen University & Research, P.O. Box 17 6700 AA Wageningen, The Netherlands

[b] Wageningen Plant Research, Wageningen University & Research, P.O. Box 16 6700 AA Wageningen, The Netherlands

Clinical Trial Registry: NCT04086108,

https://clinicaltrials.gov/ct2/show/NCT04086108

Published in Food & Function 2022, 13, 8399-8410

Abstract

Gamma-aminobutyric acid (GABA) and its precursor glutamate play signaling roles in a range of tissues. Both function as neurotransmitters in the central nervous system, but they also modulate pancreatic and immune functioning for example. Next to their endogenous production, both compounds are found in food products, reaching relatively high levels in tomato. Recent studies in rodents suggest beneficial effects of oral GABA on glucose homeostasis and blood pressure. However, the bioavailability from food remains unknown. We studied the bioavailability of GABA and glutamate from tomato relative to a solution in water. After a fasting blood sample was taken eleven healthy men randomly received 1 liter of 4 different drinks in a cross-over design with a one-week interval. The drinks were a solution of 888 mg/L GABA, a solution of 3673 mg/L glutamate, pureed fresh tomato and plain water as control. Following intake, 18 blood samples were taken at intervals for 24 hours. Plasma GABA and glutamate concentrations were determined with ultra-pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Fasting plasma GABA and glutamate concentrations were found to be 16.71 (SD 2.18) ng/mL and 4625.6 (SD 1666.1) ng/mL, respectively. Fasting GABA levels were constant (5.8 CV%) within individuals, while fasting glutamate levels varied considerably (23.5 CV%). GABA showed similar bioavailability from pureed tomato as from a solution in water. For glutamate, absorption from pureed tomato occurred more slowly as seen from a longer t_{max} (0.98±0.14 h vs 0.41±0.04 h, P = 0.003) and lower C_{max} (7815±627 ng/mL vs 16420±2778 ng/mL, P = 0.006). These data suggest that GABA is bioavailable from tomato, and that food products containing GABA could potentially induce health effects similar to those claimed for GABA supplements. The results merit further studies on the bioavailability of GABA from other food products and the health effects of GABA-rich diets.

Keywords: GABA, glutamic acid, tomato, bioavailability, kinetics, food matrix

1. Introduction

The amino acids gamma-aminobutyric acid (GABA) and its precursor glutamate are well known as primary inhibitory and excitatory neurotransmitters in the central nervous system, respectively [1]. In addition to effects on the central nervous system, GABA receptors have been identified on peripheral cells of the immune system and the pancreas [2–5]. Therefore, it has been hypothesized that GABA may also have a role in, for example, metabolic regulation of glucose homeostasis [6]. This has sparked interest in the potential beneficial effects of GABA supplements, and several rodent studies have indeed shown promising health effects of oral GABA intake. For example, GABA supplementation was found to improve diabetic symptoms (e.g. lower blood glucose levels and improved insulin sensitivity) in animal models for both type 1 and type 2 diabetes, and to lower blood pressure [7–11]. Clinical trials studying oral supplementation of GABA in humans are still scarce and focus mainly on mental aspects [12–14].

GABA is primarily known as a neurotransmitter that is endogenously formed. Endogenous GABA is synthesized from glutamate via glutamic acid decarboxylase in the brain, pancreas, liver, and it is produced by gut microbiota [15]. The GABA-precursor glutamate is produced from glutamine, a major dietary amino acid, via the action of glutaminase in the liver, brain and kidney [16]. In addition to the endogenous synthesis route, GABA and glutamate are also commonly present in the diet. Rich sources of GABA are particularly fermented foods and several fruits and vegetables [17]. Tomatoes can be considered a relevant source since they are rich in GABA, and consumption rates are high in many cultures [18]. Tomatoes are not only rich in GABA but also contain high amounts of glutamate, the natural precursor for GABA. Previous studies have demonstrated that oral GABA supplementation leads to transient peaks in circulating GABA concentrations, suggesting that oral GABA can be absorbed by the GI tract [19]. However, it has not been investigated whether GABA from a food matrix is also absorbed and capable of increasing plasma concentrations. As of yet it is also unknown whether the GABA-precursor glutamate, which is also present at high concentrations in tomatoes, can affect circulating GABA concentrations.

The potential health benefits of GABA in combination with its abundance in food sources have given rise to the speculation that GABA-rich food products like tomato may improve metabolic health [20]. The design of intervention studies that are needed to investigate this will require insight into the absorption of GABA from a food matrix compared to supplements. In view of this, a four-way crossover study was undertaken to investigate the relative oral bioavailability of GABA and its precursor glutamate from tomato compared to a solution in water in healthy volunteers.

2. Materials and methods

2.1. Subject number and characteristics

The study was carried out according to the guidelines laid out in the declaration of Helsinki and approved by the medical ethical committee of Wageningen University (METC-WU, 19/13). The study is registered at clinicaltrials.gov (NCT04086108). All participants signed a written informed consent before participating in the study. This is the first study to assess the relative bioavailability of GABA and glutamate from a food matrix, therefore sample size calculation was based on a published plasma kinetic study [19] and justified by the well-controlled nature of the current study. Sample size was estimated to be 10 participants, two additional participants were included to account for possible dropouts. We included a total of 12 healthy male participants. Exclusion criteria included suffering from any disease, any gastrointestinal disorder within 3 months prior to the intervention, recent medication or supplement use, recent substantial change in weight, adherence to a specific diet (e.g., vegetarian), using recreational drugs more than once a month, smoking and excessive alcohol consumption (>10 standardized glasses a week). Whether the participants met these criteria was assessed using a self-reported questionnaire. In addition, whether hemoglobin levels were lower than 8.5 mmol/L was measured with a fingerprick (HemoCue Hb 201, Ängelholm, Sweden) during screening in the month before the study at the Human Nutrition Research Unit of Wageningen University and Research. A total of 11 participants completed all 4 test days. One of the participants withdrew from participation for reasons that were not related to the study after the first 2 test days, and therefore his data were excluded from further analysis. More details are shown in a CONSORT flow diagram (Supplemental material 1). Participants had a mean age of 23.6 (SD 2.4) years, and mean BMI of 22.5 (SD 1.8) kg/m^2.

2.2. Study design

We conducted a randomized four-way crossover study from November to December 2019 at the Human Nutrition Research Unit of Wageningen University and Research. We assessed the relative bioavailability of GABA and glutamate from pureed tomato compared to solutions prepared in water. The study consisted of four test days for each participant with at least one-week washout in between. In a random order, participants received one of the four test products on each test day. These test products were a GABA solution in water, a glutamate solution in water, pureed tomatoes or water as a control (section 2.3).

The day before each test day, participants consumed standardized meals without high GABA containing products. In addition, they did not eat or drink anything other than water from 22:00 onwards. On the test days, participants arrived in a fasted state at the research facility. After placement of a venous catheter and donating a baseline blood sample, they received the test product. Participants were instructed to drink the allocated test product in exactly 15 minutes. Subsequently, blood samples were taken at t= 15, 30 and 45 minutes and 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 6, 7, 8, 9, 10, and

24 hours after ingestion was complete (see **Figure 1**). The venous catheter was used until t=10 hours and was kept open by slowly infusing a sterile saline solution. Samples at t=24 hours were taken by venepuncture.

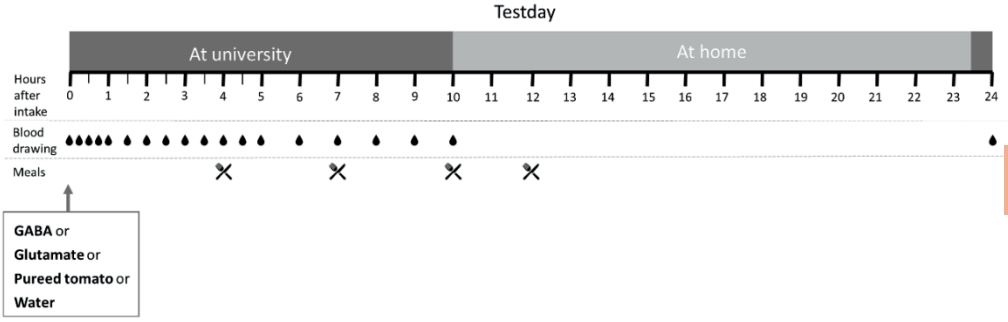


Figure 1: Study design of a single testday showing the hours after intake of the test products and the different actions of the research subjects on each day. *In this crossover design, every research subject underwent the testday four times. Each time a different test product was given.*

During the blood sampling period, participants were provided standardized meals or snacks, supplied by the university, at t= 4, 7, 10, and 12 hours. The meals were consumed after the blood sample was taken. They were low in protein to minimize glutamate intake and did not include any high GABA containing products. The composition of these meals and the food products is provided in supplemental material 2. Two hours before and until 2 hours after ingestion of the test products, the participants were not allowed to drink water. Water was provided *ad libitum* during the remainder of the test day. Between t=10 hours and t=24 hours, participants went home, but were not allowed to eat or drink anything except the provided snack at t=12 hours and water. They were also asked not to take part in intensive exercise. This was monitored by questioning the day after.

2.3. Preparation of test products

The test products were 888 mg GABA powder (99% purity, as acid, food grade, Bulksupplements, lot: 1902403) dissolved in 1 liter water, 3673 mg glutamate powder (99% purity, L-glutamic acid, food grade, Sigma Aldrich, W328512) dissolved in 1 liter water, 1 liter of pureed fresh tomatoes or 1 liter of water as control. Tomatoes (cv. *Madara, a GABA rich variety*) were provided by the vegetable breeding company Nunhems Netherlands BV (Nunhem, NL). After arrival at Wageningen University, the fresh whole (unpeeled) tomatoes were homogenized in a blender (HBH650, Hamilton Beach) for 20 seconds at maximum speed, which resulted in a homogeneous juice containing 100% tomatoes. The pureed tomato was stored in batches of 1.5 liters in plastic bags at -20 °C. A first harvest batch (~1 kilo, a week before the study start date) was processed and three samples were analyzed before the start of the study to

determine GABA and glutamate content of the tomatoes (see section 2.4). The amount of GABA and glutamate powder dissolved in water was adjusted to the amounts present in this first batch of pureed tomato. These pureed tomatoes contained 888 (SD 34.8) mg/L GABA and 3673 (SD 486) mg/L glutamate. The day before each test day, the required amount of pureed tomato was taken out of the freezer and left to thaw at room temperature. GABA and glutamate were dissolved in a liter of water in plastic bottles the day before each test day. It was specifically chosen to dissolve powders in water, instead of administering capsules, to obtain a similar concentration as was found in the freshly pureed tomato. Pureed tomato samples were taken on each test day for retrospective determination of actual GABA and glutamate contents.

2.4. Analysis of GABA and glutamate content in tomatoes

The concentrations of GABA and glutamate present in the pureed tomato were quantified using gas chromatography coupled to mass spectrometry (GCMS). The extraction procedure was according to the protocol described by Carreno-Quintero et al 2012 [21]. Briefly, the 200 mg pureed tomato fruit was extracted with 1400 µl of a 90% (v:v) methanol/water solution containing 15,000 ng/mL of GABA-d6 and 50,000 ng/mL of L-glutamic acid-d5 (both from Merck-Sigma). After sonication and centrifugation (20,000g) for each 10 minutes, 500 µL of the clear supernatant was taken and mixed with 375 µL of chloroform and 750 µL of water. After a new centrifugation (20,000g), 50 µL aliquots of the upper (polar) phase were dried overnight (16 h) by vacuum centrifugation (Savant®, SPD121P, Thermo Scientific) at room temperature, and the vials were subsequently closed under an argon atmosphere with magnetic crimp caps. Prior to their analysis, dried extracts were derivatized online using a TriPlusRSH autosampling/injection robot (Thermo Scientific), which was programmed to firstly add 12.5 µL of O-methylhydroxylamine hydrochloride (20 mg mL^{-1} pyridine), then incubate for 30 min at 40 °C with agitation, and finally derivatize with 17.5 µL of N-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) for 60 min.

The derivatized compounds were analyzed by a GCMS system (Thermo Scientific) consisting of a Trace 1300 GC with a programmable temperature vaporizing injector coupled to a TSQ8000 DUO-series triple quadrupole MS. One microliter of each sample was introduced to the injector at 70 °C using a split flow of 19 mL/min. Chromatographic separation was performed using a VF-5ms capillary column (Varian, Palo Alto, CA, USA; 30 m × 0.25 mm × 0.25 mm) including a 10 m guard column, with helium as the carrier gas at a column flow rate of 1 mL/min. The column effluent was ionized by electron impact at 70 eV and mass spectra of eluting compounds were acquired at a combined selective reaction monitoring (SRM) and full scan mode with a *m/z* range of 50 to 600 at an ion source temperature of 290 °C. Absolute levels of GABA and glutamate were calculated using calibration curves based on 6 different concentrations of GABA (from 1000 up to 100,000 ng/mL) and glutamate (from 5000 up to 500,000 ng/mL, while taking into account the recovery of their deuterated internal standards. Macronutrient composition of the pureed tomato were analyzed by

an external laboratory (Nutrilab B.V., Giessen, the Netherlands) using validated methods.

2.5. Blood collection

Blood was drawn via the catheter or via venepuncture (t=24 h samples) from a forearm vein and collected in 3 mL K_2EDTA blood collection tubes (BD Vacutainer; Becton, Dickinson and Company). Next, tubes were centrifuged at 3000*g* for 10 minutes at 4 °C (Sigma 4-16K; Sigma-zentrifugen). Plasma samples were immediately put on dry ice for rapid freezing. At the end of each test day the plasma samples were stored at -80 °C until further analysis.

2.6. Plasma sample extraction and UPLC-MS/MS analysis

Plasma GABA and glutamate concentrations were quantified according to a validated UPLC-MS/MS method. The full extraction and analysis protocol including the method validation are described elsewhere in full detail [22]. Briefly, the analytes were extracted from plasma as follows. To 100 µL of plasma, 400 µL of acetonitrile (ACN, Biosolve, Valkenswaard, the Netherlands) containing 0.1% v/v formic acid (FA, Biosolve, Valkenswaard, the Netherlands), 200 ng/mL GABA-d2 (Sigma Aldrich, Munich, Germany, 617458) and 2000 ng/mL glutamic acid-d5 (Sigma Aldrich, Munich, Germany, 631973) was added. Samples were mixed at 1400 rpm (Eppendorf thermomixer, Eppendorf) for 5 minutes and centrifuged at 15,000g for 15 minutes. The supernatant was diluted to 95% ACN with 1500 µL ACN. The extract was purified with solid phase extraction (SPE) using BondElut C8 200 mg SPE cartridges (Agilent, Santa Clara, United States). To this end, the samples were loaded onto cartridges, previously conditioned with 1 mL methanol (Biosolve, Valkenswaard, the Netherlands) and 1 mL 50% methanol. Cartridges were washed with 2 mL 95% ACN and the analytes were eluted with 2 mL 80% acetonitrile. Samples were evaporated to dryness in a vacuum concentrator (Turbovap, Biotage, Uppsala, Sweden) for 40 minutes at 30 °C and dissolved in 100µL MQ water with 0.1% v/v FA. The extracts were stored at -80 °C until analysis.

Extracts were analyzed for GABA and glutamate concentrations using an Acquity I-class UPLC coupled to a Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp, Etten-leur, the Netherlands). Chromatographic separation was performed using an Acquity UHPLC HSS reversed phase C18 column (2.1 x 150 mm with 1.8 µm particle size, Waters Corp, Etten-leur, the Netherlands). Eluent A consisted of MQ water with 0.1% v/v FA, eluent B consisted of 100% methanol. The following gradient was applied: 98% A; 0.0 – 3.0 min, 98% - 50% A; 3.0 – 6.0 min, 50% A; 6.0 – 8.0 min, 98% A; 8.0 – 10.0 min. Total runtime was 10 minutes and the eluent flowed at a rate of 0.15 mL/min. Positive electrospray ionization was used for all analytes. The MS was run in SRM mode. The following mass to charge ratios in the precursor to product ion transitions were used; for GABA 104.1>87, for GABA-d2 106.1>89.1, for glutamate 148>130 and for glutamate-d5 153>135.

Calibration ranges were 3.4 - 1500 ng/mL for GABA and 30.9 – 22,500 ng/mL for glutamate. Quantification was performed against a linear, 1/x weighted, regression curve based on the duplicate injection of calibration standards. Within- and between-day deviation of accuracy and precision of the method were < 10% at quality control concentrations of 15 ng/mL (low), 150 ng/mL (medium), and 1500 ng/mL (high) for GABA, and 1000 ng/mL (low), 5000 ng/mL (medium) and 15000 ng/mL (high) for glutamate. Similar accuracy and precision characteristics were achieved during the current analysis. Sample extraction and analysis were performed after finishing the trial, taking all samples of the same participant together in a single run. For every 10 study samples, a quality control sample was analyzed.

2.7. Data analysis

Statistical analyses were performed using R 3.6.1 [23]. Figures were produced using the R package ggplot2 [24]. The 0-10h area under the curve (AUC), the maximum concentration (C_{max}) and the time to peak (t_{max}) were calculated using GraphPad Prism 5 (GraphPad Software). For the calculation of the area under the curve the trapezoidal rule was used. Area under the curve was baseline adjusted by subtracting the area under the curve of the placebo (1 liter of tap water) condition. Normality was visually assessed using normal qqplots. For both the C_{max} of the GABA and the glutamate concentration there was one outlier, as well as for the AUC of GABA. Outliers were included in the analysis since the data is valid and outliers could be representative of the variability in the population. Furthermore, excluding these outliers did not affect the test outcome, mean, standard error and test result without the outlier are shown in supplemental material 3. The differences between the AUC, C_{max} and the t_{max} between the supplement and the tomato conditions were assessed using a paired-samples *t*-test. Differences in fasting plasma concentrations were assessed using ANOVA. Differences were considered to be significant if $P < 0.05$. Correlation between fasting plasma GABA and glutamate concentration was assessed using a Pearson's correlation. To determine the differences between individual timepoints a two-way repeated measures ANOVA was carried out in IBM SPSS Statistics 25 with Bonferroni adjustment. For this analysis the data were log-transformed, and one extreme outlier was removed.

3. Results

3.1. Participants and test products

Participant characteristics are described in the materials and methods section. A total of 11 participants completed all four test days. GABA and glutamate content of the first harvest batch of *Madara* tomatoes was analyzed just before this study, and the concentrations in the GABA and glutamate solutions were based on these analyses. The macronutrient composition of the pureed *Madara* tomato is shown in **Table 1.** As a check to assess the actual administered dose, a sample from the pureed tomato was taken at each study day, just before it was consumed. These pureed samples were analyzed after completion of the study for their GABA and glutamate content. In the pureed tomato samples (n=8), the GABA content was on average 1044 (SD 98.5, i.e., 9.4 CV%) mg/L which is 118% of the initially measured 888 mg/L, and the glutamate content was on average 3574 (SD 297, i.e., 8.3 CV%) mg/L which is 97% of the initial 3673 mg/L. These differences between actual and target values, which were < 20%, were deemed acceptable in light of the 20% tolerance limits that are used for e.g. nutrient declaration [25].

Table 1: macronutrient composition of the pureed *Madara* tomato.

Macronutrients	g/100g pureed tomato
Water	**91.9**
Protein	**1.1**
Fat	**<0.5**
Carbohydrate	**3.7**
Fibre	**1.7**

[1] a litre of pureed tomato weighs 1.03 kilogram

3.2. Plasma kinetics of GABA and glutamate

The mean plasma GABA concentration-time profiles following consumption of each test product are shown in **Figure 2A**. Descriptive kinetic parameters are summarized in **Table 2** and individual data are presented in **Figure 3**. Following consumption of the GABA solution in water and pureed tomato, GABA concentrations peaked rapidly after 0.5 and 0.36 hours, respectively. Participants tended to have a shorter mean GABA t_{max} after consumption of pureed tomato (P = 0.052). Although the mean AUC and C_{max} of GABA were higher following ingestion of pureed tomato, they were not significantly different. The difference in the mean AUC and C_{max} between the GABA and pureed tomato can mostly be explained by a single outlier; one participant has a more than 3-fold higher AUC and 5-fold higher Cmax of GABA in response to tomato consumption as compared to the GABA solution in water (**Figure 3A and B**).

Table 2: plasma kinetic parameters of GABA and glutamate. *Parameters are calculated from plasma kinetics after ingestion of the GABA and glutamate solutions and pureed tomato. The 10-hour area under the curve shown is corrected for the area under the curve after taking placebo.*

	Test product				
	GABA solution		Pureed tomato		
GABA	Mean	SEM	Mean	SEM	P-value
C_{max} (ng/mL)	74.7	10.5	184.0	88.4	0.213
t_{max} (h)	0.50	0.05	0.36	0.04	0.052
AUC (ng/mL^{-1}.h)	59.7	10.1	115.7	40.0	0.117

	Glutamate solution		Pureed tomato		
Glutamate	Mean	SEM	Mean	SEM	P-value
C_{max} (ng/mL)	16420	2779	7815	628	0.006
t_{max} (h)	0.41	0.04	0.98	0.14	0.003
AUC (ng/mL^{-1}.h)	7579	2272	7362	1873	0.929

Differences in mean concentration at the individual timepoints were assessed with a two-way repeated measures ANOVA. When comparing the GABA concentration in plasma following consumption of the GABA solution to pureed tomato, a significant difference was only seen at 2 hours after intake (19.74±0.65 ng/mL vs 23.08±1.19 ng/mL, P=0.003) (**Figure 2A)**. When comparing to water, significant differences in GABA plasma concentrations were found until 4 hours after consumption of both the pureed tomato and the GABA solution (t=4 hours: 19.06±1.24 ng/mL and 19.23±0.59 ng/mL vs 16.11±0.57 ng/mL, P=0.015 and P=0.010 respectively). After the GABA solution, the GABA plasma concentration was elevated compared to water at two later timepoints as well; after 5 hours (16.19±0.41 vs 14.07±0.61 ng/mL, P=0.003) and 6 hours (16.04±0.54 vs 14.26±0.48 ng/mL, P=0.003). Although some differences in plasma kinetics were observed, on average, the food matrix did not markedly affect GABA bioavailability.

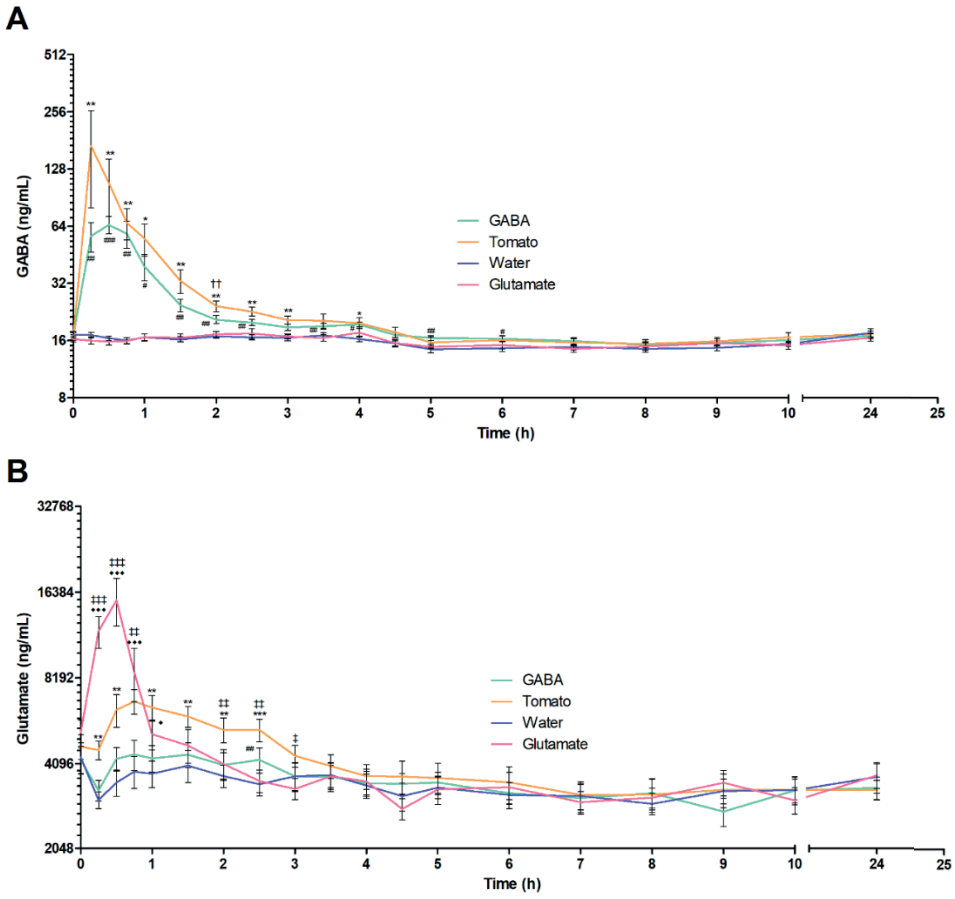


Figure 2: Average plasma GABA and glutamate concentration-time profiles for 11 volunteers. *A) Average plasma GABA concentration over time is shown after ingestion of GABA solution (888 mg in 1 liter), pureed tomato (1 liter, 1044 mg GABA and 3574 mg glutamate), water (1 liter) or glutamate (3673 mg in 1 liter). B) Average plasma glutamate concentration over time is shown after ingestion of GABA (888 mg in 1 liter), tomato (1 liter), water (1 liter) or glutamate (3673 mg in 1 liter). Data is presented as mean ± SEM. The following symbols represent significant differences in GABA/glutamate plasma concentration for the test products relative to that after ingestion of water. For GABA, #: P<0.05; ##: P <0.01; ###: P<0.001. For tomato, *: P<0.05; **: P<0.01; ***: P<0.001. For glutamate, ♦: P<0.05; ♦♦: P<0.01; ♦♦♦: P<0.001. Significant differences between the GABA solution and the pureed tomato are represented by: ††: P <0.01. Significant differences between the glutamate solution and the pureed tomato are represented by: ‡: P<0.05; ‡‡: P<0.01; ‡‡‡: P<0.001.*

The mean plasma glutamate concentration-time profiles after ingestion of each test product are shown in **Figure 2B**. Following consumption of the glutamate solution, glutamate plasma levels peaked rapidly. However, the mean glutamate C_{max} was significantly lower after the consumption of pureed tomato compared to the glutamate solution (7815±627 ng/mL vs 16420±2778 ng/mL, P = 0.006) (**Table 2**). In addition, the glutamate curve shows a significantly longer t_{max}, after the consumption of pureed tomato compared to the solution in water (0.41±0.04 h vs. 0.98±0.14 h, P = 0.003). Yet, when comparing to the glutamate solution, the average AUC of glutamate after pureed tomato was not significantly different.

When comparing the glutamate plasma concentration at individual timepoints following consumption of the glutamate solution and the pureed tomato, significant differences were seen at: 15 minutes (11933±1508 vs 4571±346, P=<0.0001), 30 minutes (15379±2925 vs 6330±838, P=<0.0001), 45 minutes (8579±1818 vs 6776±669, P=0.001), 2 hours (4082502 vs 5379±541, P=0.003), 2,5 hours (3563±323 vs 5378±488, P=0.001) and 3 hours (3339±284 vs 4365±387, P=0.022) (**Figure 2B**). When comparing to water, significant differences were found until 1 hour (t=1 hour: 5195±619 vs 3775±400, P=0.030) after the consumption of the glutamate solution and for 2.5 hours (t= 2.5 hours: 5378±487 vs 3470±314, P=<0.0001) after the consumption of pureed tomato. In summary, these results show a lower and later peak glutamate plasma concentration and a slower time-dependent decline after the ingestion of pureed tomato as compared to the glutamate solution.

Figure 2 also shows that glutamate intake does not seem to influence GABA plasma concentrations. While this is mostly also true the other way around, a significantly elevated glutamate concentration as compared to water is observed 2.5 hours after intake of the GABA solution (4047±433 vs 3705±332, P=0.010).

3.3. Interindividual differences in kinetic parameters

The interindividual differences were illustrated using line graphs, comparing the GABA and glutamate kinetic variables of the tomato and the solutions (**Figure 3**). For both GABA and glutamate concentration on average no significant differences in AUC were found (**Table 2**), while interindividual differences are seen in these linegraphs. Eight out of the eleven participants showed a higher AUC for GABA after taking the pureed tomato in comparison to the GABA solution (**Figure 3A**). The t_{max} and C_{max} of GABA also show interindividual differences (**Figure 3B, C**). In the case of glutamate, eight out of the eleven participants showed a lower AUC after taking the pureed tomato in comparison to the glutamate solution (**Figure 3D**). The direction of the differences in the t_{max} and C_{max} of glutamate show more consistency between the participants (**Figure 3E, F**).

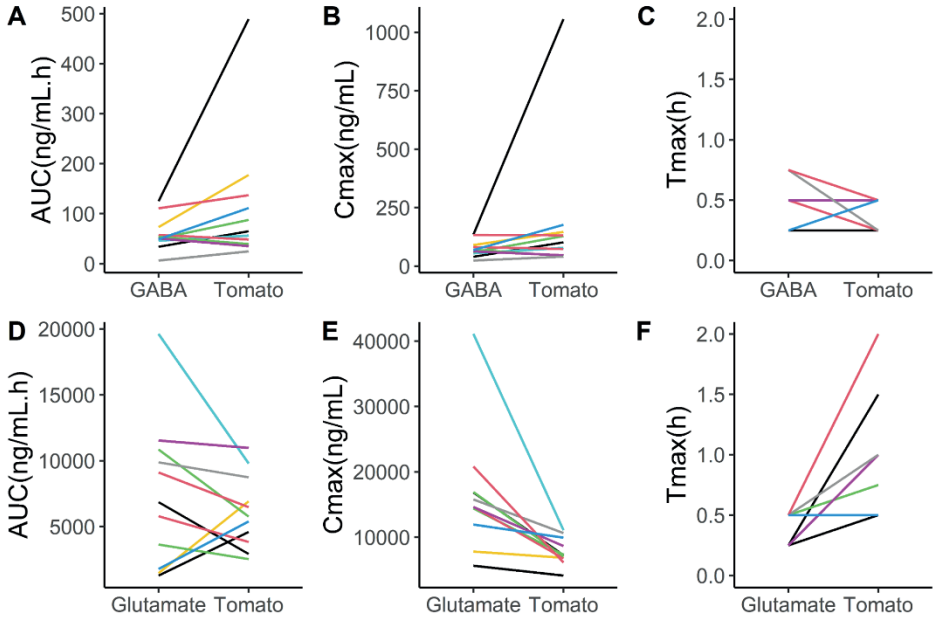


Figure 3: Line graphs showing kinetic parameters per individual, comparing tomato to the GABA and glutamate solutions. *The individual's GABA kinetic parameters 10-hour AUC, C_{max} and t_{max} are depicted in respectively Figure A, B and C. The individual's glutamate kinetic parameters 10-hour AUC, C_{max} and t_{max} are depicted in respectively Figure D, E and F. Colors represent the research subjects.*

3.4. Variation in fasting GABA and glutamate concentrations

Fasting GABA and glutamate levels were measured 4 times in each individual over the course of 5 weeks, each time in the early morning. **Figure 4** shows the 4 GABA and glutamate plasma concentrations per individual. We found no correlation between fasting GABA and glutamate plasma concentrations, r = 0.137. Average fasting GABA was found to be 16.71 (SD 2.18) ng/mL in a range from 14.2 – 20.3 ng/mL (**Figure 4A**). Average fasting glutamate was found to be 4626 (SD 1666) ng/mL in a range from 3021 – 6723 ng/mL (**Figure 4B**).

The average within person coefficient of variation (CV) for GABA was 5.8% (SD 2.58%). The average within person CV for glutamate was 23.5% (SD 9.76%). This shows that fasting GABA concentrations are fairly stable over a period of 5 weeks, while this is less the case for glutamate. With an ANOVA it was assessed whether the observed differences between individuals in fasting plasma concentrations were not due to chance. Fasting plasma GABA ($F(10, 33) = 14.41$, $p =<0.001$) and glutamate

(F(10, 33) = 4.84, p = <0.001) concentrations were found to be significantly different between participants.

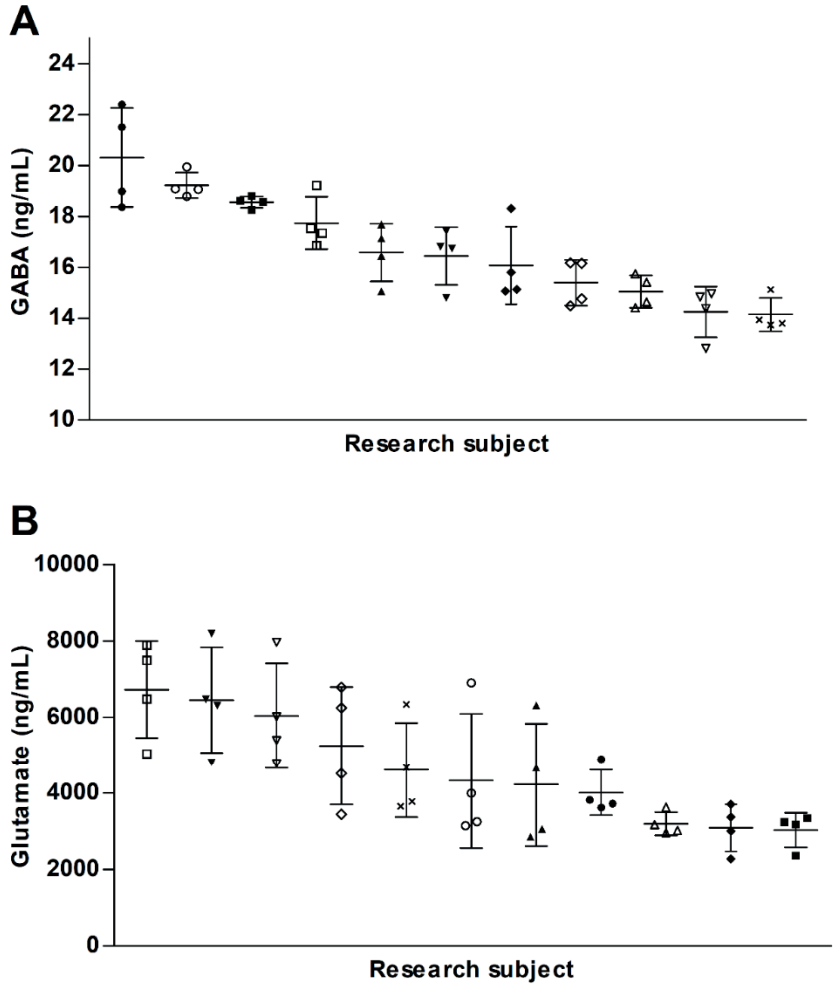


Figure 4: Scatter dot plots of fasting GABA and glutamate concentrations from 11 volunteers. *Showing a) GABA and b) glutamate fasting plasma concentrations in ng/mL per individual measured at 4 different days with at least a week in between. Concentrations are ordered from highest on the left to lowest on the right. In both graph A and B, the different symbols indicate the same participant. Expressed as individual values and mean ± SD.*

4. Discussion

In view of their pleiotropic effects, including but not limited to their role as neurotransmitters, more insight into the uptake and potential effects of GABA and glutamate obtained through the diet is of relevance. However, thus far limited information is available about the oral bioavailability of GABA and glutamate. To our knowledge we are the first to show that GABA is bioavailable from tomato and that its bioavailability is not markedly hampered by the food matrix, although some differences in kinetics are observed. On the other hand, we show that glutamate kinetics are significantly influenced by the tomato matrix.

The plasma kinetics of oral GABA have not extensively been investigated since it is primarily known for its endogenous properties as neurotransmitter and not as a nutritional component. We show that after oral GABA intake, the plasma GABA concentration peaks rapidly after 0.5 hours, reaching values of ~4.5 times those at baseline. A similar study was done by Li et al. (2015), who describes the plasma kinetics of a GABA supplement [19]. Our findings are not entirely in line with their results. The authors also observe a rapid increase in GABA levels after intake. However, they measured a time to peak concentration of 1.5 hours at ~45 times the baseline values. Remarkably, the participants received a single dose of 2 grams of GABA, which is only 2.25 times the dose that was given in the present study. Formulation in tablets could have been responsible for a longer time to peak by requiring solubilization of GABA, as was observed in the study by Li et al. (2015). In addition, compared to administration in a relatively large volume of water, higher local concentrations are likely when taking a tablet. Combined with the higher dose administered, this may at least partly explain the difference found in maximum achieved concentration.

In both our results and the paper from Li et al. (2015), a clear transient elevation in plasma GABA concentration is observed after ingestion of GABA. However, measurements in blood only reflect the current circulating concentrations, which are the balance of various processes that may work in opposing directions. These processes such as the absorption, distribution, metabolism and excretion of GABA are not yet fully understood. Studies in mice suggest that intravenously administered GABA is distributed extremely rapidly (within 3 minutes after injection), mainly to the liver and kidney, and metabolized to other components, like succinate [26–28]. In the liver, GABA-transaminase activity is high and it is therefore mainly responsible for the catabolism of GABA [29]. The high uptake of GABA from the portal vein into the liver would keep the plasma GABA concentration in systemic circulation relatively low. Studies with, for example, isotopically labeled GABA would therefore be necessary to further elucidate the route that GABA takes after oral ingestion.

The main goal of the present study was to determine the relative bioavailability of GABA from tomato. We show that GABA uptake and bioavailability from tomato is not significantly hampered by the food matrix. Therefore, it can be concluded that the food matrix does not markedly affect absorption of GABA, although small differences in

kinetics are observed. Interindividual variation in GABA concentration after ingestion of tomato is larger as compared to GABA in a solution. In some individuals, the matrix seems to enhance the GABA bioavailability, even when considering the slightly higher amount of GABA in the pureed tomato (118%) compared to the GABA solution. For further research a larger sample size would be needed to study the incidence of these differences. In the current study, the tomatoes were pureed to minimize interindividual variation. This standardizes the mode of ingestion and avoids batch effects. This pre-processing may have influenced the bio-accessibility of GABA. Ingestion of whole tomatoes would require additional steps in the digestive process which could possibly delay or inhibit GABA absorption. Therefore, it is not unlikely that GABA bioavailability from whole tomatoes is slightly lower.

Other nutrients or non-nutritive components present in the pureed tomato do not seem to influence GABA bioavailability. GABA is hydrophilic in nature and hence its absorption is unlikely to be affected by the presence of fats which is for example the case for the carotenoids in tomato [30]. Data from other studies have shown that GABA uptake from the intestinal lumen requires active transport processes, such as through PAT1 (SLC36A1) which is a low affinity and high capacity transporter (in mM range) [31]. PAT1 is also responsible for the transport of other small amino acids like proline, alanine and glycine, and of pharmacological GABA analogues like vigabatrin [32]. Literature regarding these GABA analogues may be relevant in interpreting our findings. The kinetic properties of vigabatrin have been more extensively investigated than those of GABA itself [33]. Nøhr et al. (2014) looked at the interactions with food with regard to the uptake of vigabatrin [33]. They found that the presence of amino acids that are substrates of PAT1 (proline, tryptophan and sarcosine) inhibit the uptake of vigabatrin. Further research could investigate whether this is also the case for GABA uptake. In tomato these amino acids are also present but in much lower amounts compared to GABA [34]. Therefore, the results of this study do not rule out potential effects of food components, not present in tomato, on GABA bioavailability. Studies have been performed with functional foods that aimed for high GABA concentrations, like fermented milk products [35,36]. Additional specific assessment of GABA bioavailability in these type of food products would be relevant.

Notwithstanding the consistency of our findings when considering these at a group level, it remains of interest to pay attention to specifically the interindividual variation in GABA kinetics. Just like Li et al. (2015) we find a large interindividual variation in GABA plasma kinetics (AUC CV of 81% (Li et. al (2015)) and 56% (present study)). Many factors influence plasma kinetics, like for example gastric emptying rate which has an interindividual variation of 16% to 39% (AUC CV) even under highly standardized conditions [37]. However, genetic polymorphisms in transport processes can also play a role here. Genetic variation in genes encoding for proteins involved in GABA transport are known to exist. Single nucleotide polymorphisms (rs357629, rs357618) in the PAT1 transporter have been found to affect its function for instance [38]. Similarly, a single nucleotide polymorphism (rs555044) was found to influence

the function of GAT2 (SLC6A13), a transporter protein in the liver, responsible for GABA transport [39].

Next to GABA, tomatoes are also very rich in glutamate, one of the other main neurotransmitters and the precursor of GABA. For this reason, we also investigated the relative oral bioavailability of glutamate from tomato and any interaction with GABA levels. Although we do not see an increase in GABA concentration after the ingestion of glutamate, we do observe a slight increase in glutamate concentration after the intake of GABA. This observation needs independent replication before any speculation on the mechanism is warranted. Glutamate is well known as a nutrient and food additive, therefore more research about its oral bioavailability is already available. We found that glutamate appears rapidly in the bloodstream, peaking after about 25 minutes at ~3.6 times the baseline concentration. Multiple studies have been conducted that investigated the plasma kinetics of monosodium glutamate (MSG) [40,41]. In those studies, a 3 times higher dose was used compared to the present study. Both in our study as in those studies the glutamate was given as a solution in water, although in a lower volume (~300 mL). Considering this higher dose, their results are comparable to the glutamate concentration time curve that we have measured. They report a similarly rapid increase in glutamate plasma concentrations with a peak after about 30 minutes at ~7.6 and ~14.6 times the baseline concentration. Glutamate normally has a low bioavailability, with 95% of the glutamate ingested being used as substrate by the enterocytes or metabolized into other amino acids like alanine, aspartate and GABA [42,43]. Stegink et al. (1983) showed that the bioavailability of glutamate is even further reduced by the co-ingestion of carbohydrates [40]. In their study, a high oral dose of glutamate together with 1.1 gram of carbohydrate led to a blunted peak of the glutamate concentration. The authors suggest that the carbohydrates serve as a source of pyruvate which would facilitate the catabolism of glutamate into alanine and therefore reduces the amount of glutamate that reaches the circulation. Also for other amino acids it is known that co-ingestion with carbohydrates reduces their bio-availability [44]. This would be in line with our findings. The amount (1 kg) of pureed tomato that the participants consumed contained 37 grams of carbohydrates. It is therefore likely that also in our study the carbohydrates reduced the maximum concentration of glutamate in the circulation.

On the other hand, as opposed to the findings by Stegink et al. (1983), we show that the relative oral bioavailability, as indicated by the AUC, did not differ between the glutamate solution and the pureed tomato. However, these two conditions cannot directly be compared since tomato also contains protein-bound glutamate, while the dose of the glutamate solution was based solely on the amount of free glutamate in the tomatoes. The amount of pureed *Madara* tomato consumed contained 11 grams of protein, and on average 7 – 13% of plant protein consists of glutamate [45,46]. This would mean that upon ingestion of the pureed tomato the participants received an estimated maximum of 1430 mg protein-bound glutamate in addition to the measured

3574 mg of free glutamate. Glutamate plasma kinetics following tomato consumption has not been investigated previously, but presumably the proteins that contain glutamate need to be (fully) hydrolyzed before absorption. The plasma kinetics of glutamate from tomato show a similar pattern as is commonly seen with amino acids that are incorporated in proteins, like phenylalanine and leucine [47]. This leads to a prolonged absorption phase, as shown by elevated plasma concentrations until 2.5 hours after ingestion of pureed tomato as opposed to just 1 hour after ingestion of the glutamate solution. We therefore speculate that the glutamate AUC would have been lower after ingestion of pureed tomato if no glutamate-containing protein was present. This strongly suggests that the relative oral bioavailability of free glutamate is lower after the ingestion of pureed tomato as compared to a glutamate solution in water, most likely due to the presence of carbohydrates in tomato.

The crossover design also gave us the opportunity to measure fasting GABA and glutamate. This could provide us with useful information since several studies suggest that their concentrations in the circulation are associated with a spectrum of central nervous system related outcomes. For example, abnormal GABA and glutamate concentrations in blood have been associated with depression, autism, schizophrenia and bipolar disorder[48–59]. For each individual, we collected 4 samples at least one week apart. Their analyses show the individual variation of GABA and glutamate plasma concentrations over time. The GABA plasma concentration appears to be very stable. Both within each individual on different days and within the day, while there are significant differences between individuals. Petty et. al, (1983) related GABA plasma concentration to the occurrence of depression. They found a similar range of GABA plasma concentrations of up to 20 ng/mL as we found here. Interestingly, GABA concentrations below 10 ng/mL were only found in depressed individuals. As of yet, it is unclear how genetic variability, differences in microbiota composition [60–62], or long-term dietary intake of GABA, influence the plasma concentrations of individuals. As compared to GABA, fasting glutamate plasma concentrations between the different days were found to be far more variable. It is unknown how differences in behavior and food intake in between the test days might have influenced glutamate concentrations. However, the participants were asked to eat the same meals the day before each test day. These findings should be considered when interpreting research that investigates the potential use of glutamate as biomarker.

We show that a food matrix has a different impact on GABA and glutamate bioavailability, and the practical implications of these results are therefore different for both molecules. Since the glutamate kinetic profile is influenced by the food matrix it seems that physiological effects of glutamate supplementation cannot be immediately generalized to glutamate that is ingested with food. On the other hand, food products, or at least tomato, are a viable natural source of GABA. Bioavailability does not seem to be reduced by the food matrix. In light of these findings, it is tempting to speculate that the presumed positive health effects ascribed to GABA, would also be applicable to GABA-containing food products. While long term health effects of a high GABA intake

have not yet been determined in humans, the results of this study merit further investigation of the potential health effects of GABA-rich foods and diets.

References

[1] S.E. Hyman, Neurotransmitters, Current Biology. 15 (2005) R154–R158. https://doi.org/10.1016/J.CUB.2005.02.037.

[2] A. Barragan, J.M. Weidner, Z. Jin, E.R. Korpi, B. Birnir, GABAergic signalling in the immune system, Acta Physiologica. 213 (2015) 819–827. https://doi.org/10.1111/apha.12467.

[3] E. Xu, M. Kumar, Y. Zhang, W. Ju, T. Obata, N. Zhang, S. Liu, A. Wendt, S. Deng, Y. Ebina, M.B. Wheeler, M. Braun, Q. Wang, Intra-islet insulin suppresses glucagon release via GABA-GABAA receptor system, Cell Metab. 3 (2006) 47–58. https://doi.org/10.1016/J.CMET.2005.11.015.

[4] N. Ben-Othman, A. Vieira, M. Courtney, F. Record, E. Gjernes, F. Avolio, B. Hadzic, N. Druelle, T. Napolitano, S. Navarro-Sanz, S. Silvano, K. Al-Hasani, A. Pfeifer, S. Lacas-Gervais, G. Leuckx, L. Marroquí, J. Thévenet, O.D. Madsen, D.L. Eizirik, H. Heimberg, J. Kerr-Conte, F. Pattou, A. Mansouri, P. Collombat, Long-Term GABA Administration Induces Alpha Cell-Mediated Beta-like Cell Neogenesis, Cell. 168 (2017) 73-85.e11. https://doi.org/10.1016/J.CELL.2016.11.002.

[5] D. Menegaz, D.W. Hagan, J. Almaça, C. Cianciaruso, R. Rodriguez-Diaz, J. Molina, R.M. Dolan, M.W. Becker, P.C. Schwalie, R. Nano, F. Lebreton, C. Kang, R. Sah, H.Y. Gaisano, P.-O. Berggren, S. Baekkeskov, A. Caicedo, E.A. Phelps, Mechanism and effects of pulsatile GABA secretion from cytosolic pools in the human beta cell, Nat Metab. 1 (2019) 1110–1126. https://doi.org/10.1038/s42255-019-0135-7.

[6] G.M. Noguchi, M.O. Huising, Integrating the inputs that shape pancreatic islet hormone release, Nat Metab. 1 (2019) 1189–1201. https://doi.org/10.1038/s42255-019-0148-2.

[7] J. Tian, H. Dang, Z. Chen, A. Guan, Y. Jin, M.A. Atkinson, D.L. Kaufman, γ-aminobutyric acid regulates both the survival and replication of human β-cells, Diabetes. 62 (2013) 3760–3765. https://doi.org/10.2337/db13-0931.

[8] K. Hayakawa, M. Kimura, K. Kamata, Mechanism underlying γ-aminobutyric acid-induced antihypertensive effect in spontaneously hypertensive rats, Eur J Pharmacol. 438 (2002) 107–113. https://doi.org/10.1016/S0014-2999(02)01294-3.

[9] S. Sohrabipour, M.R. Sharifi, A. Talebi, M. Sharifi, N. Soltani, GABA dramatically improves glucose tolerance in streptozotocin-induced diabetic rats fed with high-fat diet, Eur J Pharmacol. 826 (2018) 75–84. https://doi.org/10.1016/J.EJPHAR.2018.01.047.

[10] N. Soltani, H. Qiu, M. Aleksic, Y. Glinka, F. Zhao, R. Liu, Y. Li, N. Zhang, R. Chakrabarti, T. Ng, T. Jin, H. Zhang, W.-Y. Lu, Z.-P. Feng, G.J. Prud'homme, Q. Wang, GABA exerts protective and regenerative effects on islet beta cells and

reverses diabetes., Proc Natl Acad Sci U S A. 108 (2011) 11692–7. https://doi.org/10.1073/pnas.1102715108.

[11] N. Rathwa, N. Parmar, S.P. Palit, R. Patel, R.S. Bhaskaran, A.V. Ramachandran, R. Begum, Calorie restriction potentiates the therapeutic potential of GABA in managing type 2 diabetes in a mouse model, Life Sci. 295 (2022) 120382. https://doi.org/10.1016/J.LFS.2022.120382.

[12] A.M. Abdou, S. Higashiguchi, K. Horie, M. Kim, H. Hatta, H. Yokogoshi, Relaxation and immunity enhancement effects of gamma-aminobutyric acid (GABA) administration in humans., Biofactors. 26 (2006) 201–8.

[13] L. Steenbergen, R. Sellaro, A.-K. Stock, C. Beste, L.S. Colzato, γ-Aminobutyric acid (GABA) administration improves action selection processes: a randomised controlled trial, Sci Rep. 5 (2015) 12770. https://doi.org/10.1038/srep12770.

[14] A. Yoto, S. Murao, M. Motoki, Y. Yokoyama, N. Horie, K. Takeshima, K. Masuda, M. Kim, H. Yokogoshi, Oral intake of γ-aminobutyric acid affects mood and activities of central nervous system during stressed condition induced by mental tasks, Amino Acids. 43 (2012) 1331–1337. https://doi.org/10.1007/s00726-011-1206-6.

[15] S.B. Sarasa, R. Mahendran, G. Muthusamy, B. Thankappan, D.R.F. Selta, J. Angayarkanni, A Brief Review on the Non-protein Amino Acid, Gamma-amino Butyric Acid (GABA): Its Production and Role in Microbes, Curr Microbiol. 77 (2020) 534–544. https://doi.org/10.1007/S00284-019-01839-W/FIGURES/5.

[16] J.M. Matés, J.A. Segura, J.A. Campos-Sandoval, C. Lobo, L. Alonso, F.J. Alonso, J. Márquez, Glutamine homeostasis and mitochondrial dynamics, Int J Biochem Cell Biol. 41 (2009) 2051–2061. https://doi.org/10.1016/J.BIOCEL.2009.03.003.

[17] B. Redruello, A. Szwengiel, V. Ladero, B. del Rio, M.A. Alvarez, Identification of technological/metabolic/environmental profiles of cheeses with high GABA contents, LWT. (2020) 109603. https://doi.org/10.1016/J.LWT.2020.109603.

[18] B. Burton-Freeman, K. Reimers, Tomato Consumption and Health: Emerging Benefits, Am J Lifestyle Med. 5 (2011) 182–191. https://doi.org/10.1177/1559827610387488.

[19] J. Li, Z. Zhang, X. Liu, Y. Wang, F. Mao, J. Mao, X. Lu, D. Jiang, Y. Wan, J.-Y. Lv, G. Cao, J. Zhang, N. Zhao, M. Atkinson, D.L. Greiner, G.J. Prud'homme, Z. Jiao, Y. Li, Q. Wang, Study of GABA in Healthy Volunteers: Pharmacokinetics and Pharmacodynamics, Front Pharmacol. 6 (2015) 260. https://doi.org/10.3389/fphar.2015.00260.

[20] D.H. Ngo, T.S. Vo, An Updated Review on Pharmaceutical Properties of Gamma-Aminobutyric Acid, Molecules. 24 (2019). https://doi.org/10.3390/MOLECULES24152678.

[21] N. Carreno-Quintero, A. Acharjee, C. Maliepaard, C.W.B. Bachem, R. Mumm, H. Bouwmeester, R.G.F. Visser, J.J.B. Keurentjes, Untargeted metabolic quantitative

trait loci analyses reveal a relationship between primary metabolism and potato tuber quality, Plant Physiol. 158 (2012) 1306–1318. https://doi.org/10.1104/pp.111.188441.

[22] T.H. de Bie, R.F. Witkamp, M.A. Jongsma, M.G.J. Balvers, Development and validation of a UPLC-MS/MS method for the simultaneous determination of gamma-aminobutyric acid and glutamic acid in human plasma, J Chromatogr B Analyt Technol Biomed Life Sci. 1164 (2021) 122519. https://doi.org/10.1016/j.jchromb.2020.122519.

[23] R Core Team, R: A Language and Environment for Statistical Computing., (2017).

[24] H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer New York, New York, NY, 2009. https://doi.org/10.1007/978-0-387-98141-3.

[25] European Commission, Guidance Document for Competent Authorities for the Control of Compliance with EU Legislation on Regulation (EU) No 1169/2011, Council Directive 90/496/EEC and Directive 2002/46/EC with Regard to the Setting of Tolerances for Nutrient Values Declared on a Label., (2012).

[26] E. Kuroda, M. Watanabe, T. Tamayama, M. Shimada, Autoradiographic distribution of radioactivity from 14C-GABA in the mouse, Microsc Res Tech. 48 (2000) 116–126. https://doi.org/10.1002/(SICI)1097-0029(20000115)48:2<116::AID-JEMT7>3.0.CO;2-P.

[27] D. Ravasz, G. Kacso, V. Fodor, K. Horvath, V. Adam-Vizi, C. Chinopoulos, Catabolism of GABA, succinic semialdehyde or gamma-hydroxybutyrate through the GABA shunt impair mitochondrial substrate-level phosphorylation, Neurochem Int. 109 (2017) 41–53. https://doi.org/10.1016/j.neuint.2017.03.008.

[28] A. Mori, M. Kosaka, Incorporation γ-aminobutyric acid (GABA) in the brain and internal organs of mice, Psychiatry Clin Neurosci. 15 (1961) 92–97. https://doi.org/10.1111/j.1440-1819.1961.tb00637.x.

[29] P. Ferenci, T. Stehle, J. Ebner, R. Schmid, D. Häussinger, Uptake and catabolism of γ-aminobutyric acid by the isolated perfused rat liver, Gastroenterology. 95 (1988) 402–407. https://doi.org/10.1016/0016-5085(88)90497-0.

[30] P. Riso, A. Brusamolino, L. Scalfi, M. Porrini, Bioavailability of carotenoids from spinach and tomatoes., Nutr Metab Cardiovasc Dis. 14 (2004) 150–6. https://doi.org/10.1016/s0939-4753(04)80035-8.

[31] M. Boll, M. Foltz, C.M.H. Anderson, C. Oechsler, G. Kottra, D.T. Thwaites, H. Daniel, Substrate recognition by the mammalian proton-dependent amino acid transporter PAT1, Mol Membr Biol. 20 (2003) 261–269. https://doi.org/10.1080/0968768031000100759.

[32] L. Metzner, K. Neubert, M. Brandsch, Substrate specificity of the amino acid transporter PAT1, Amino Acids. 31 (2006) 111–117. https://doi.org/10.1007/s00726-005-0314-6.

[33] M.K. Nøhr, S. Frølund, R. Holm, C.U. Nielsen, Pharmacokinetic aspects of the anti-epileptic drug substance vigabatrin: focus on transporter interactions, Ther Deliv. 5 (2014) 927–942. https://doi.org/10.4155/tde.14.55.

[34] D. Mallamace, C. Corsaro, A. Salvo, N. Cicero, A. Macaluso, G. Giangrosso, V. Ferrantelli, G. Dugo, A multivariate statistical analysis coming from the NMR metabolic profile of cherry tomatoes (The Sicilian Pachino case), Physica A: Statistical Mechanics and Its Applications. 401 (2014) 112–117. https://doi.org/10.1016/J.PHYSA.2013.12.054.

[35] D.M. Linares, T.F. O'Callaghan, P.M. O'Connor, R.P. Ross, C. Stanton, Streptococcus thermophilus APC151 Strain Is Suitable for the Manufacture of Naturally GABA-Enriched Bioactive Yogurt, Front Microbiol. 7 (2016) 1876. https://doi.org/10.3389/fmicb.2016.01876.

[36] K. Pouliot-Mathieu, C. Gardner-Fortier, S. Lemieux, D. St-Gelais, C.P. Champagne, J.-C. Vuillemard, Effect of cheese containing gamma-aminobutyric acid-producing lactic acid bacteria on blood pressure in men, PharmaNutrition. 1 (2013) 141–148. https://doi.org/10.1016/J.PHANU.2013.06.003.

[37] M. Grimm, M. Koziolek, J.P. Kühn, W. Weitschies, Interindividual and intraindividual variability of fasted state gastric fluid volume and gastric emptying of water, European Journal of Pharmaceutics and Biopharmaceutics. 127 (2018) 309–317. https://doi.org/10.1016/j.ejpb.2018.03.002.

[38] P. Crocco, E. Hoxha, S. Dato, F. De Rango, A. Montesanto, G. Rose, G. Passarino, Physical decline and survival in the elderly are affected by the genetic variability of amino acid transporter genes., Aging. 10 (2018) 658–673. https://doi.org/10.18632/aging.101420.

[39] B. Yu, Y. Zheng, D. Alexander, A.C. Morrison, J. Coresh, E. Boerwinkle, Genetic Determinants Influencing Human Serum Metabolome among African Americans, PLoS Genet. 10 (2014) e1004212. https://doi.org/10.1371/journal.pgen.1004212.

[40] L.D. Stegink, L.J. Filer, G.L. Baker, Effect of carbohydrate on plasma and erythrocyte glutamate levels in humans ingesting large doses of monosodium L-glutamate in water., Am J Clin Nutr. 37 (1983) 961–8. https://doi.org/10.1093/ajcn/37.6.961.

[41] T.E. Graham, V. Sgro, D. Friars, M.J. Gibala, Glutamate ingestion: the plasma and muscle free amino acid pools of resting humans, American Journal of Physiology-Endocrinology and Metabolism. 278 (2000) E83–E89. https://doi.org/10.1152/ajpendo.2000.278.1.E83.

[42] P.J. Reeds, D.G. Burrin, B. Stoll, F. Jahoor, Intestinal Glutamate Metabolism, J Nutr. 130 (2000) 978S-982S. https://doi.org/10.1093/jn/130.4.978S.

[43] S.P. Haÿs, J.M. Ordonez, D.G. Burrin, A.L. Sunehag, Dietary Glutamate Is Almost Entirely Removed in Its First Pass Through the Splanchnic Bed in Premature Infants, Pediatr Res. 62 (2007) 353–356. https://doi.org/10.1203/PDR.0b013e318123f719.

[44] S.H.M. Gorissen, N.A. Burd, H.M. Hamer, A.P. Gijsen, B.B. Groen, L.J.C. van Loon, Carbohydrate Coingestion Delays Dietary Protein Digestion and Absorption but Does Not Modulate Postprandial Muscle Protein Accretion, J Clin Endocrinol Metab. 99 (2014) 2250–2258. https://doi.org/10.1210/jc.2013-3970.

[45] S.H. Choi, D.S. Kim, N. Kozukue, H.J. Kim, Y. Nishitani, M. Mizuno, C.E. Levin, M. Friedman, Protein, free amino acid, phenolic, β-carotene, and lycopene content, and antioxidative and cancer cell inhibitory effects of 12 greenhouse-grown commercial cherry tomato varieties, Journal of Food Composition and Analysis. 34 (2014) 115–127. https://doi.org/10.1016/j.jfca.2014.03.005.

[46] S.H.M. Gorissen, J.J.R. Crombag, J.M.G. Senden, W.A.H. Waterval, J. Bierau, L.B. Verdijk, L.J.C. van Loon, Protein content and amino acid composition of commercially available plant-based protein isolates, Amino Acids. 50 (2018) 1685–1695. https://doi.org/10.1007/s00726-018-2640-5.

[47] R. Koopman, S. Walrand, M. Beelen, A.P. Gijsen, A.K. Kies, Y. Boirie, W.H.M. Saris, L.J.C. van Loon, Dietary Protein Digestion and Absorption Rates and the Subsequent Postprandial Muscle Protein Synthetic Response Do Not Differ between Young and Elderly Men, J Nutr. 139 (2009) 1707–1713. https://doi.org/10.3945/jn.109.109173.

[48] H.-L. Cai, R.-H. Zhu, H.-D. Li, X.-H. Zhang, L. Hu, W. Yang, H.-S. Ye, Elevated plasma γ-aminobutyrate/glutamate ratio and responses to risperidone antipsychotic treatment in schizophrenia, Prog Neuropsychopharmacol Biol Psychiatry. 34 (2010) 1273–1278. https://doi.org/10.1016/J.PNPBP.2010.07.006.

[49] A. Arrúe, R. Dávila, M. Zumárraga, N. Basterreche, M.A. González-Torres, B. Goienetxea, M.I. Zamalloa, J.B. Anguiano, J. Guimón, GABA and Homovanillic Acid in the Plasma of Schizophrenic and Bipolar I Patients, Neurochem Res. 35 (2010) 247–253. https://doi.org/10.1007/s11064-009-0048-z.

[50] F. Petty, G.L. Kramer, M. Fulton, F.G. Moeller, A.J. Rush, Low Plasma GABA Is a Trait-Like Marker for Bipolar Illness, Neuropsychopharmacology. 9 (1993) 125–132. https://doi.org/10.1038/npp.1993.51.

[51] E. Küçükibrahimoğlu, M.Z. Saygın, M. Çalışkan, O.K. Kaplan, C. Ünsal, M.Z. Gören, The change in plasma GABA, glutamine and glutamate levels in fluoxetine- or S-citalopram-treated female patients with major depression, Eur J Clin Pharmacol. 65 (2009) 571–577. https://doi.org/10.1007/s00228-009-0650-7.

[52] F. Petty, G.L. Kramer, M. Fulton, L. Davis, A.J. Rush, Stability of plasma GABA at four-year follow-up in patients with primary unipolar depression, Biol Psychiatry. 37 (1995) 806–810. https://doi.org/10.1016/0006-3223(94)00226-S.

[53] D. Dhossche, H. Applegate, A. Abraham, P. Maertens, L. Bland, A. Bencsath, J. Martinez, Elevated plasma gamma-aminobutyric acid (GABA) levels in autistic youngsters: stimulus for a GABA hypothesis of autism., Med Sci Monit. 8 (2002)

PR1-6. http://www.ncbi.nlm.nih.gov/pubmed/12165753 (accessed August 27, 2019).

[54] A.J. Russo, Correlation between Hepatocyte Growth Factor (HGF) and Gamma-Aminobutyric Acid (GABA) Plasma Levels in Autistic Children, Biomark Insights. 8 (2013) BMI.S11448. https://doi.org/10.4137/BMI.S11448.

[55] J.S. Kim, W. Schmid-Burgk, D. Claus, H.H. Kornhuber, Increased serum glutamate in depressed patients, Archiv For Psychiatrie Und Nervenkrankheiten. 232 (1982) 299–304. https://doi.org/10.1007/BF00345492.

[56] C. Shimmura, S. Suda, K.J. Tsuchiya, K. Hashimoto, K. Ohno, H. Matsuzaki, K. Iwata, K. Matsumoto, T. Wakuda, Y. Kameno, K. Suzuki, M. Tsujii, K. Nakamura, N. Takei, N. Mori, Alteration of Plasma Glutamate and Glutamine Levels in Children with High-Functioning Autism, PLoS One. 6 (2011) e25340. https://doi.org/10.1371/journal.pone.0025340.

[57] H. Mitani, Y. Shirayama, T. Yamada, K. Maeda, C.R. Ashby, R. Kawahara, Correlation between plasma levels of glutamate, alanine and serine with severity of depression, Prog Neuropsychopharmacol Biol Psychiatry. 30 (2006) 1155–1158. https://doi.org/10.1016/J.PNPBP.2006.03.036.

[58] Z. Zheng, T. Zhu, Y. Qu, D. Mu, Blood Glutamate Levels in Autism Spectrum Disorder: A Systematic Review and Meta-Analysis, PLoS One. 11 (2016) e0158688. https://doi.org/10.1371/journal.pone.0158688.

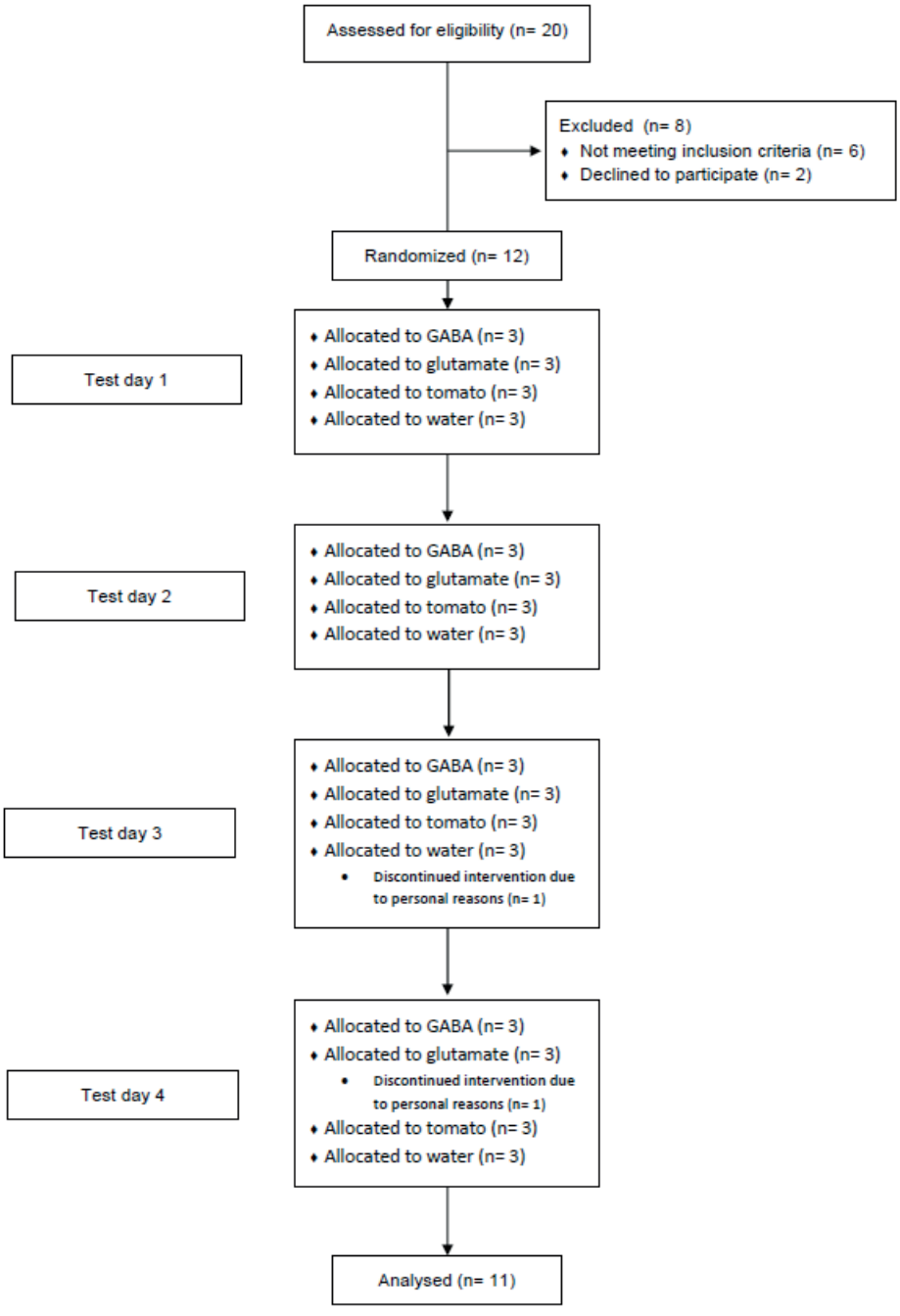
[59] A. Palomino, A. González-Pinto, A. Aldama, C. González-Gómez, F. Mosquera, G. González-García, C. Matute, Decreased levels of plasma glutamate in patients with first-episode schizophrenia and bipolar disorder, Schizophr Res. 95 (2007) 174–178. https://doi.org/10.1016/J.SCHRES.2007.06.012.

[60] M. Matsumoto, T. Ooga, R. Kibe, Y. Aiba, Y. Koga, Y. Benno, Colonic Absorption of Low-Molecular-Weight Metabolites Influenced by the Intestinal Microbiome: A Pilot Study., PLoS One. 12 (2017) e0169207. https://doi.org/10.1371/journal.pone.0169207.

[61] E. Barrett, R.P. Ross, P.W. O'Toole, G.F. Fitzgerald, C. Stanton, γ-Aminobutyric acid production by culturable bacteria from the human intestine, J Appl Microbiol. 113 (2012) 411–417. https://doi.org/10.1111/j.1365-2672.2012.05344.x.

[62] P. Strandwitz, K.H. Kim, D. Terekhova, J.K. Liu, A. Sharma, J. Levering, D. McDonald, D. Dietrich, T.R. Ramadhar, A. Lekbua, N. Mroue, C. Liston, E.J. Stewart, M.J. Dubin, K. Zengler, R. Knight, J.A. Gilbert, J. Clardy, K. Lewis, GABA-modulating bacteria of the human gut microbiota, Nat Microbiol. 4 (2018) 396. https://doi.org/10.1038/s41564-018-0307-3.

Supplemental material 1: CONSORT flow diagram showing recruitment, inclusion, randomization and lost to follow-up.



Supplemental material 2: Composition of standardized meals A) the day before each test day and B) on each test day. The day before each test day the participants were not limited to specific times to eat the products. Participants were received either a large (L) or an extra-large (XL) diet, depending on their energy requirement.

A

Products	Unit	L (n=7)	XL (n=4)
Whole wheat bread	Slice	8	10
Light butter	10 gr	4	5
Cheese spread	15 gr	2	3
Nutella	15 gr	2	3
Strawberry jam	15 gr	2	2
Paté	15 gr	2	2
Granola with yoghurt and fruit	170 gr	1	1
Orange juice	200 mL	1	1
Semi-skimmed milk	200 mL	1	2
Apple	1	1	1
Tangerine	1	2	2
Nuts	30 gr	1	1
Tandoori chicken	450 gr	1	1
Custard	135 mL	2	2

B

Products	Unit	L (n=7)	XL (n=4)
t=4			
Low-protein bread	35 gr	3	4
Light butter	10 gr	1	2
Fruit sprinkles	20 gr	2	2
Nutella	15 gr	1	2
Ice tea	200 mL	1	1
Apple	1	1	1
t=7			
Apple	1	1	1
Soft drink (Dubbelfris)	200 mL	1	1
t=10			
Nasi goreng (rice, ham, leek, egg)	grams	550	650
Skimmed yoghurt with fruit taste	grams	175	200
t=12			
Soft drink (Coca Cola/Sinas)	330 mL	1	1

Supplemental material 3: plasma kinetic parameters of GABA excluding the outlier. Parameters are calculated from plasma kinetics after ingestion of the GABA solution and pureed tomato. The 10-hour area under the curve shown is corrected for the area under the curve after taking placebo.

	Test product				
	GABA solution		Pureed tomato		
GABA	Mean	SEM	Mean	SEM	P-value
C_{max} (ng/mL)	68.6	9.4	84.1	12.4	0.074
t_{max} (h)	0.52	0.04	0.38	0.04	0.051
AUC (ng/mL^{-1}.h)	53.1	8.4	78.3	15.7	0.061

Chapter 5

Effects of GABA supplementation on glucose control in adults with prediabetes: A double-blind, randomized, placebo-controlled trial

Authors: Tessa H. de Bie[a, b], Renger F. Witkamp[a], Michiel G.J. Balvers[a], Maarten A. Jongsma[b]

Affiliations:

[a] Division of Human Nutrition and Health, Wageningen University & Research, P.O. Box 17, 6700 AA Wageningen, The Netherlands

[b] Wageningen Plant Research, Wageningen University & Research, P.O. Box 16, 6700 AA Wageningen, The Netherlands

Under revision at the American Journal of Clinical Nutrition

Abstract

Background: Gamma-aminobutyric acid (GABA) is mainly known as an endogenously produced neurotransmitter. However, GABA intake from dietary sources like tomatoes and fermented foods can be significant. Studies in rodent models have shown beneficial effects of oral GABA supplementation on glucose homeostasis and cardiovascular health. Still, it is currently unknown whether oral GABA supplementation produces cardiometabolic benefits in humans.

Objectives: This study aimed to investigate whether oral GABA supplementation can improve glucose homeostasis in individuals at risk of developing type 2 diabetes.

Methods: In a randomized, placebo-controlled, double-blind, parallel-arm trial, 52 individuals with prediabetes (classified by impaired glucose tolerance and/or impaired fasting glucose), aged 50-70 years old and a BMI ≥25 kg/m2, received either 500 mg GABA 3 times daily, or a placebo, for 95 days. The primary outcome was the effect of the intervention on glucose response after an oral glucose tolerance test. As exploratory secondary outcomes, parameters of glycemic control (HbA1c, insulin, glucagon, mean amplitude of glycemic excursions, and standard deviation as measured with flash glucose monitoring), cardiovascular health (blood pressure, 24-hour blood pressure, circulating triglycerides, cholesterol) and self-reported sleep quality were measured before and after the intervention.

Results: Compared with placebo, GABA supplementation for 95 days did not change the postprandial glucose response. All other parameters (including fasting plasma GABA concentration) showed no significant effects of the GABA intervention at a group level after correction for the false discovery rate.

Conclusions: GABA supplementation did not change the postprandial glucose response in individuals at risk of developing type 2 diabetes. Notwithstanding this outcome, findings in secondary outcome measures and considering that the participants were not yet in an advanced state of prediabetes warrant further research in individuals with diabetes and other cardiometabolic disorders.

Registered at www.clinicaltrials.gov as NCT04303468.

Keywords: GABA, prediabetes, overweight, blood pressure, sleep, metabolic health, glucose, insulin, glucagon, triglycerides

Abbreviations:
FGM: flash glucose monitoring, MAGE: mean amplitude of glycemic excursions, ABPM: ambulatory blood pressure monitor, PSQI: Pittsburgh sleep quality index.

1. Introduction

Gamma-aminobutyric acid (GABA) is best known as an inhibitory neurotransmitter in the central nervous system. However, significant amounts of GABA are also present in non-neuronal peripheral tissues in concentrations up to micromolar levels [1,2]. In line with this, GABA receptors of both the $GABA_A$ (ionotropic) and $GABA_B$ (metabotropic) class have been identified on many non-neuronal cell types, including cells of the immune system, pancreas, liver, adipose tissue, ovaries, adrenal glands, intestinal tract, and the kidney [3,4]. Interestingly, GABA is also present in the human diet and produced by the gut microbiota [5]. Relatively rich sources of GABA are tomatoes (up to 1.9 g/kg), melons (up to 3 g/kg), and fermented food products (up to 10 g/kg in matured cheeses) [6–8]. Functional foods with added GABA have also been developed, and recently a GABA-rich tomato was created in Japan as the first CRISPR-edited food on the market [9].

GABA is also produced in the beta-cells of the pancreas and increasing evidence indicates an important role for GABA in the paracrine and autocrine regulation of insulin and glucagon release [10]. In line with this, pancreatic islets from patients with diabetes are GABA-depleted, suggesting that disturbed GABA signaling may play a role in the disease [11]. Several studies have explored the effects of exogenous GABA administration in animal models of both type 1 and type 2 diabetes [12–15]. These studies have demonstrated that GABA, administered orally or intravenously for 5 to 16 weeks, can improve insulin sensitivity and glucose tolerance. The proposed underlying mechanisms include a reduction in low-grade inflammation, which often accompanies (pre-)diabetes, and an increase in pancreatic beta-cell mass.

Short-term studies into GABA's potential beneficial metabolic effects in humans have also been published. In a study by Li et al. [16], healthy participants received supplements containing 2 grams of GABA as a single dose or three times a day for a week. The authors showed that GABA is taken up in the blood when taken orally and promotes the production of insulin and glucagon in healthy adults (16). In a recent study, we showed that GABA is also bioavailable from dietary sources (tomatoes), producing a sharp but relatively short-lasting (<5h) elevation in plasma GABA concentration after tomato consumption [17]. Therefore, GABA-rich food sources could potentially play a relevant role in preventing type 2 diabetes development.

The prediabetic stage offers an important window of opportunity to prevent further advancement to clinical diabetes [18]. Individuals with prediabetes are characterized by impaired glucose tolerance and/or elevated fasting glucose. Reversal of prediabetes to normal glucose homeostasis leads to a 56% lower future diabetes risk [19]. Given the evidence from rodent studies, we hypothesize that GABA intake may affect diabetes development. However, so far, it is unclear whether acute or long-term intake of GABA can benefit individuals with prediabetes since appropriate intervention studies are lacking to translate these findings to humans.

Therefore, we performed a randomized, placebo-controlled, double-blind, parallel-arm trial investigating the potential beneficial effects of 95 days of oral GABA supplementation on individuals with prediabetes. The intervention was designed to study the post-intervention effects of GABA on the postprandial glucose response. It also included secondary outcome measures to explore the effects of GABA on other parameters of metabolic health, such as blood pressure, markers of cardiovascular health, circulating GABA concentrations, and sleep quality. At the start of the intervention, the acute effects of GABA on the postprandial glucose response and blood pressure were also assessed.

2. Methods

The study was approved by the medical ethical committee of Wageningen University and is registered at clinicaltrials.gov under NCT04303468. The study was performed according to the Declaration of Helsinki (20) and took place from September 2020 to September 2021 in the Human Nutrition Research Unit of Wageningen University and Research (the Netherlands).

2.1. Study population

Males and females aged 50 to 70, with a BMI above or equal to 25 kg/m2, were recruited from an area in and around Wageningen, the Netherlands. An in-house database, flyers, and social media advertisements were all used to reach a sufficient number of responding potential participants. Oral and written information was provided before written informed consent was obtained. Participants were invited to the research facility for a screening session where they underwent an oral glucose tolerance test (OGTT) with 75gr glucose (Beldico, Glucomedics). Blood was drawn with a fingerprick (Freestyle Freedom Lite, Abbott) to assess blood glucose concentration at fasting, 1 hour and 2 hours after the glucose drink. Participants with impaired fasting glucose (fasting glucose ≥ 6.1 and ≤ 6.9 mmol/L) and/or impaired glucose tolerance (glucose levels ≥8.6 mmol/L, 1 hour after an OGTT or/and glucose levels ≥ 7.8 and ≤ 11.1 mmol/L, 2-hours after an OGTT) were included. At screening, participants were also weighed, and their veins were checked for suitability to draw blood. Participants were excluded from participation according to the following criteria (as assessed by questionnaire): diagnosed with or treated for diabetes; having other conditions that could influence the study results, like liver, pancreatic, cardiovascular, gastro-intestinal, or endocrine diseases; use of medication or supplements that could affect the study results, in particular, glucose-regulating drugs or drugs interacting with GABA receptors (e.g., benzodiazepines); sensitivity to medical skin adhesives; more than 5 kg weight change in the past 12 weeks; excessive alcohol consumption (>21 glasses/week on average for males and >14 glasses/week for females); employment by Wageningen University, division Human Nutrition and Health; participation in another trial in the preceding three months.

2.2. Study design and intervention

The study was designed as a randomized, placebo-controlled, double-blind, parallel-arm trial (Figure 1A). Participants received three times a day for 95 days (~3 months), either 500 mg GABA capsules (1500 mg/day in total, Swanson Health) or placebo capsules (microcrystalline cellulose, JRS). Participants were asked to take a capsule before each main meal. The study was double-blind; the study team and participants were unaware of the group allocation. The capsules looked identical and were coded by a researcher not involved in the study. Block randomization was also conducted by

a researcher not involved in the study. Stratification was based on BMI (below and above 35 kg/m2) and sex.

Baseline and outcome measures were assessed during measurement periods (Figure 1). As a primary outcome, the postprandial glucose response was evaluated on day -3 of the measurement period. As exploratory secondary outcomes, we also measured glycemic variability, postprandial insulin and glucagon response, HbA1c, blood pressure, 24h blood pressure, blood lipids, sleep quality, and GABA and glutamate plasma concentrations. In addition, at the start of the intervention, the acute effects of GABA on the postprandial glucose response and blood pressure were assessed (days 1 and 2). An overview of the activities during the measurement periods is shown in Figure 1B. During the intervention period, the participants received new capsules every month. At these monthly visits, the capsules were counted to assess compliance. In addition, participants received a telephone call one week after the start of the intervention to increase compliance.

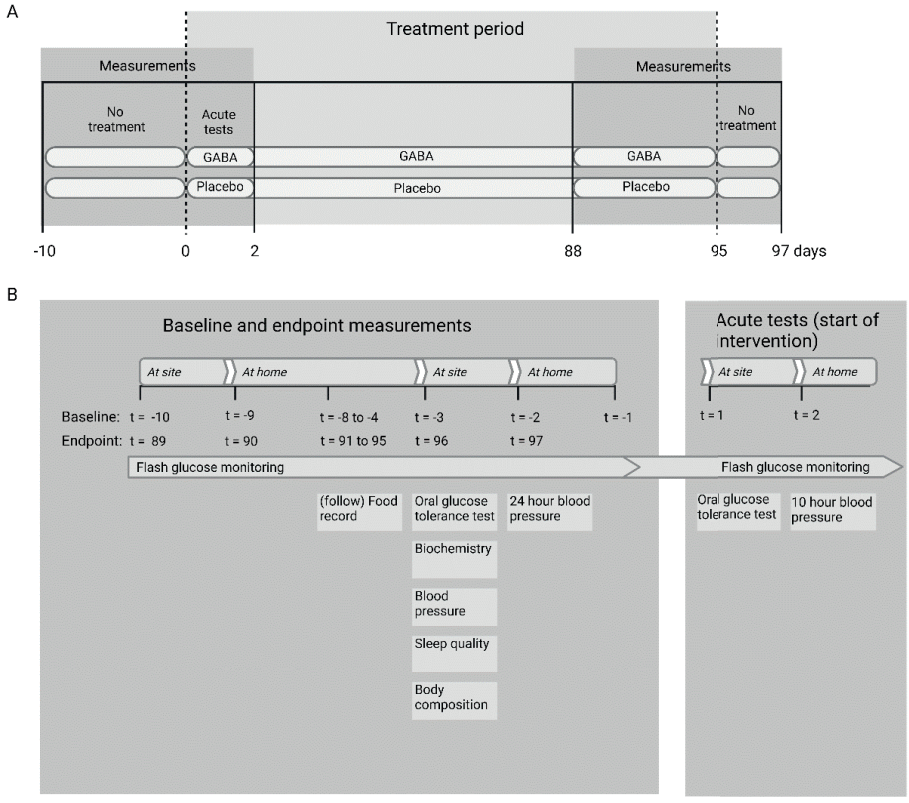


Figure 1: Flowchart showing A) the parallel study design consisting of measurements and a treatment period and B) an overview of measurements that were done at baseline and at the end of the intervention to assess the long-term and acute effects of 3dd 500 mg GABA on different health parameters. *The numbers represent the days of the study, with the treatment starting on day 1 until day 95. On day -10 until day -1 measurements are done before the intervention and on day 96 and 97 measurements are done after the intervention and no treatment is given. Measurement period at baseline: day -10: flash glucose sensor is placed; day -8 to -4: food diary is recorded; day -3: pre-intervention oral glucose tolerance test, blood pressure measurement and sleep quality questionnaire; day -2: pre-intervention 24-hour blood pressure. Acute effects: day 1: oral glucose tolerance test, while GABA/placebo is already taken; day 2: 10-hour blood pressure monitoring, while GABA/placebo is taken. Endpoint measurements: day 89: flash glucose sensor is placed; day 91 to 95: The diet follows the recorded food diaries; day 96: post-intervention oral glucose tolerance test, blood pressure measurement and sleep quality questionnaire; day 97: post-intervention 24-hour blood pressure.*

2.3. Diet standardization

During the intervention, the participants were asked not to change their habitual food intake and physical activity level substantially. Participants were asked to record a food diary in the five days (days -8 to -4, figure 1) before the baseline measurement on day -3 (figure 1). At the end of the intervention, participants were asked to repeat (the same meals at the same times) this recorded diet again the five days (days 91 to 95, figure 1) before the final OGTT on day 96 (figure 1). We introduced this matching of the diet before the OGTT to accurately measure changes in glycemic variability with flash glucose monitoring and reduce within-person variability in the OGTT results. The evening before the OGTT, participants also consumed a standardized meal provided by the university (622 kcal, 16.4 gr fat, 94 gr carbohydrate, 11.4 gr fiber, 19 gr protein), and they were not allowed to eat or drink anything other than water after 22:00. Furthermore, they were asked to refrain from strenuous exercise that evening. Consumption of alcohol and recreational drugs was not allowed for at least 48 hours before the OGTT. On the morning of the OGTT, the participant's compliance with these guidelines was recorded.

2.4. Oral glucose tolerance test and biochemical analysis

Postprandial glucose response was assessed by an OGTT in which 75 gr glucose was consumed with 200 mL water (Beldico, Glucomedics). A peripheral venous catheter was inserted into an antecubital vein 30 minutes before the first blood drawing. Blood was drawn at fasting (21 mL) and 30, 60, and 120 minutes (9 mL) after the glucose drink. Blood tubes (Vacutainers, BD) containing sodium fluoride were used to analyze glucose, and blood tubes containing lithium heparin were used to analyze glucagon, insulin, triglycerides, LDL, and HDL cholesterol. From these tubes, plasma was prepared by centrifugation at 3000g for 8 minutes at 20°C. Blood tubes containing EDTA were used to analyze GABA and glutamate, which were centrifuged at 3000g for 10 minutes at 4°C. After separation, plasma samples were stored at -80°C until the completion of the study. Glucose, glucagon, and insulin were measured at all time points of the OGTT. Concentrations of glycated hemoglobin (HbA1c, measured in whole EDTA blood analyzed within three days from blood drawing), triglycerides, LDL and HDL cholesterol, GABA, and glutamate were measured in the fasting blood sample. Glucagon (#81520, Crystal Chem) and adiponectin (#DRP300, R&D systems) were measured with an ELISA kit according to the manufacturer's protocol. GABA and glutamate concentrations in plasma were determined using a previously validated UPLC-MS/MS method described in full detail elsewhere [20]. In short, GABA and glutamate were extracted from plasma with acetonitrile containing deuterated GABA and glutamate as internal standards. The extracts were further purified using solid phase extraction, after which they were analyzed on a triple quadrupole mass spectrometer operated in positive electrospray ionization. Concentrations of GABA and glutamate were calculated against calibration standards that ranged between 3.4 to 2500 ng/mL and 30.9 ng/mL to 22,500 ng/mL, respectively. This method is sufficiently

sensitive and robust to detect endogenous circulating GABA [17,20]. All other biochemical analyses (e.g., HbA1c, glucose, insulin, lipids, AST/ALT(to rule out hepatotoxicity)) were performed at the Clinical Chemistry and Hematology Laboratory of Gelderse Vallei Hospital (Ede, the Netherlands) using standard clinical laboratory assays.

2.5. Flash glucose monitoring

Participants were provided a factory-calibrated flash glucose monitoring system (FGM, FreeStyle Libre, Abbott) for the entire measurement period (days -10 to -1 and 89 to 97). The system measures interstitial glucose concentrations every 15 minutes. The sensor was attached to the upper arm of the participants, and scanning the sensor to allow data transfer, was required at least once every 8 hours. Only the data collected during the five days participants recorded a food diary was used to analyze glycemic variability. From this data, the standard deviation and the mean amplitude of glycemic excursions (MAGE) were calculated according to previously described methods using the R environment for statistical computing version 4.0.2 (https://www.R-project.org) [21]. Participants with less than three days of available data were excluded from the analysis.

2.6. Blood pressure

Blood pressure was measured two times (HEM-907 IntelliSense, OMRON) after 5 minutes of rest in a fasted condition. A third measurement was done if the first two measurements varied by more than 10 mmHg; all measurements were averaged. In addition to these separate measurements, 24-hour blood pressure (ABPM-50, Contec) was measured by the participants at home the day after the OGTT, starting at 9:00 and ending at 9:00 the next morning. Blood pressure was measured every 30 minutes during the day (09:00 to 21:00) and every hour during the night (01:00 to 6:00). For both blood pressure measurements, the cuff was placed on the non-dominant arm. Participants with less than 70% available data were excluded from the analysis of 24-hour blood pressure data only.

2.7. Other outcomes

The Pittsburgh Sleep Quality Index (PSQI) was used to assess self-reported sleep quality over the last month. The questionnaire was filled out during the OGTT. The score was calculated as described in the literature [22]. With the PSQI, participants can score 0 to 21 points; a higher score indicates poorer sleep quality. Weight and waist circumference were also measured on the same day as the OGTT but in a fasted state.

2.8. Acute effects of GABA

The participants started with the intervention three days after finishing the baseline measurements (day 1, figure 1). On the first day of the intervention, the participants underwent another OGTT to assess the potential acute effects of GABA. The first GABA (500 mg) or placebo capsule was taken with the glucose drink. During the OGTT, the glucose levels were assessed with flash glucose monitoring (section 2.5). On the second day of the intervention, participants wore an ambulatory blood pressure monitor (ABPM-50, Contec) for 10 hours while taking their GABA or placebo capsules at prespecified times: 9:00, 13:00, and 18:00.

2.9. Statistical analysis

The sample size of this study was based on the postprandial glucose response as the primary outcome. A sample size of 46 participants would provide a power of 80% with an α of 0.05 to detect an additive difference of 17%. This estimated effect size and standard deviation were based on intervention effects from literature, using the glucose concentration 60 minutes after an OGTT as a proxy for the postprandial glucose response [23]. The sample size was increased by 10% to 52 participants to account for possible dropouts.

All outcomes assessed more than once in a single day (glucose, insulin, glucagon, 24-hour blood pressure, and the acute effects of GABA) were statistically evaluated with linear mixed models using IBM SPSS Statistics version 25. The fasting concentration was subtracted from all subsequent values for that day (delta) to determine the effects on the postprandial response. Treatment and time were added as fixed effects with the pre-intervention (baseline) values as a covariate. The participant identifier was added as random effect. Time (factor variable) was set as the repeated variable, and the covariance structure was set to AR1. For all linear mixed models, it was tested whether there was a significant interaction effect with treatment and time and treatment and baseline values. For none of the outcomes, this was the case. Therefore, the interaction term was not added to the model.

For the other outcomes (HbA1c, fasting glucose, insulin, and glucagon, mean glucose, mean amplitude of glycemic excursions, and standard deviation as measured with flash glucose monitoring, HOMA-IR, blood pressure, fasting triglycerides, cholesterol, adiponectin, GABA and glutamate, weight, waist circumference, AST/ALT and self-reported sleep quality), a one-way ANCOVA was performed using the R environment for statistical computing version 4.0.2 (https://www.R-project.org). The linear models (lm) function was used for the statistical analysis, with the formula: "post-intervention value" ~ "treatment" + "pre-intervention value". The respective baseline value was added as a covariate. The distribution of the residuals was checked for normality by examining Q-Q plots. In the cases where the residuals were not normally distributed, the data were logarithmically transformed (insulin, HOMA-IR, triglycerides).

Homoscedasticity was assessed by plotting the residuals against the fitted values. Outliers that varied more than three standard deviations from the residuals were removed from the analysis. If this was the case (PSQI score), it is mentioned in the results section. The interaction term "treatment*pre-intervention value" was checked for significance to determine the homogeneity of the regression slopes. In the case of a significant interaction, this was further investigated using Johnson-Neyman plots [24]. For creating these plots, the R function "johnson_neyman" from the package "interactions" was used [25]. As recommended, the outcomes of the Johnson-Neyman plots were adjusted for multiple testing [26]. Estimates and 95% confidence intervals of the interaction effect are reported.

The outcomes of the statistical assessments were reported as estimates with 95% confidence intervals and p-value (significant if < 0.05). P-values were also adjusted for the false discovery rate with the R function "p.adjust" using the Benjamini-Hochberg method [27]. Figures were produced using the R package "ggplot2" [28]. Figure of the study design was created with BioRender.com.

3. Results

3.1. Study population

Fifty-two participants were randomly assigned to receive either the GABA or the placebo-containing capsules. Forty-nine participants completed the intervention (Figure 2). Three participants did not complete the intervention due to perceived adverse effects (tiredness), an inability to honor commitments, and difficult venous access. One participant completed only the secondary outcomes due to difficult venous access at the end of the intervention. Table 1 displays the baseline characteristics of the 49 participants that completed the intervention. Notably, the percentage of females included in the study was relatively low (34.7%). Based on capsule count, compliance was high in both groups (GABA: 94% ± 7.4%, placebo: 97% ± 3.8%).

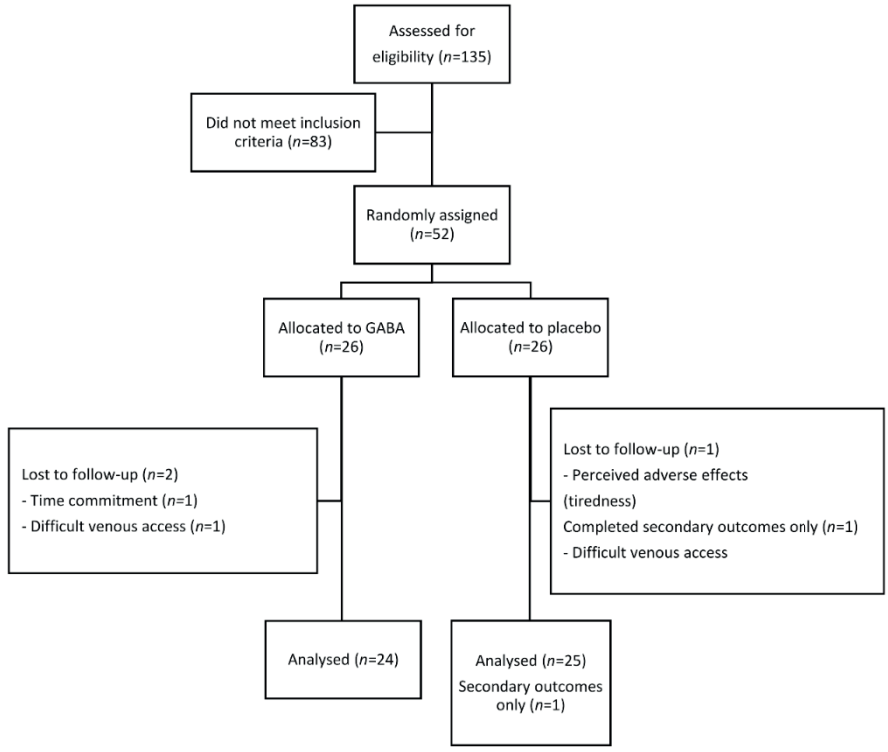


Figure 2: Flowchart showing number of participants that were excluded, included, randomized and lost to follow-up in the trial.

Table 4: General baseline characteristics and medication use of participants that completed the study[1]

	Total	**GABA**	**Placebo**
n	49	24	25
Sex = female (%)	17 (34.7%)	9 (37.5%)	8 (32.0%)
Age, y	65 [13.0]	62 [12.3]	65 [9.0]
BMI, kg/m²	28.0 [2.7]	27.6 [2.8]	28.4 [2.7]
Waist circumference, mm	1044 ± 78	1044 ± 82	1044 ± 75
Blood pressure medication = yes (%)	4 (8.2%)	1 (4.2%)	3 (12.0%)
Cholesterol medication = yes (%)	4 (8.2%)	2 (8.3%)	2 (8.0%)
Anti-inflammatory medication = yes (%)	8 (16.3%)	5 (20.8%)	3 (12.0%)
Chronically	5 (10.2%)	4 (16.7%)	1 (4.0%)
Sporadically	3 (6.1%)	1 (4.2%)	2 (8.0%)

[1] Values are mean ± SD if normally distributed and otherwise presented as median [IQR]

3.2. Effects of GABA on glycemic control

Parameters of glycemic control were assessed at baseline and the end of the intervention. No significant group differences were found in the postprandial glucose response after an OGTT (0.21 mmol/L; 95% CI: -0.252, 0.674; P: 0.364). Also, the insulin (0.01 µIU/mL; 95% CI: -0.059, 0.086; P: 0.711) and glucagon (0.59 pg/mL; 95% CI: -1.30, 2.48; P: 0.531) response did not show any significant differences (Figure 3). However, there was a significant reduction in fasting glucose concentration after the GABA intervention compared to the placebo group (Table 2). However, this was no longer a significant finding after correction for the false discovery rate. No significant effects were found for HbA1c or parameters of glycemic variability as measured with flash glucose monitoring during a 5-day period. Effects on fasting insulin, glucagon, and HOMA-IR also did not differ significantly between the groups.

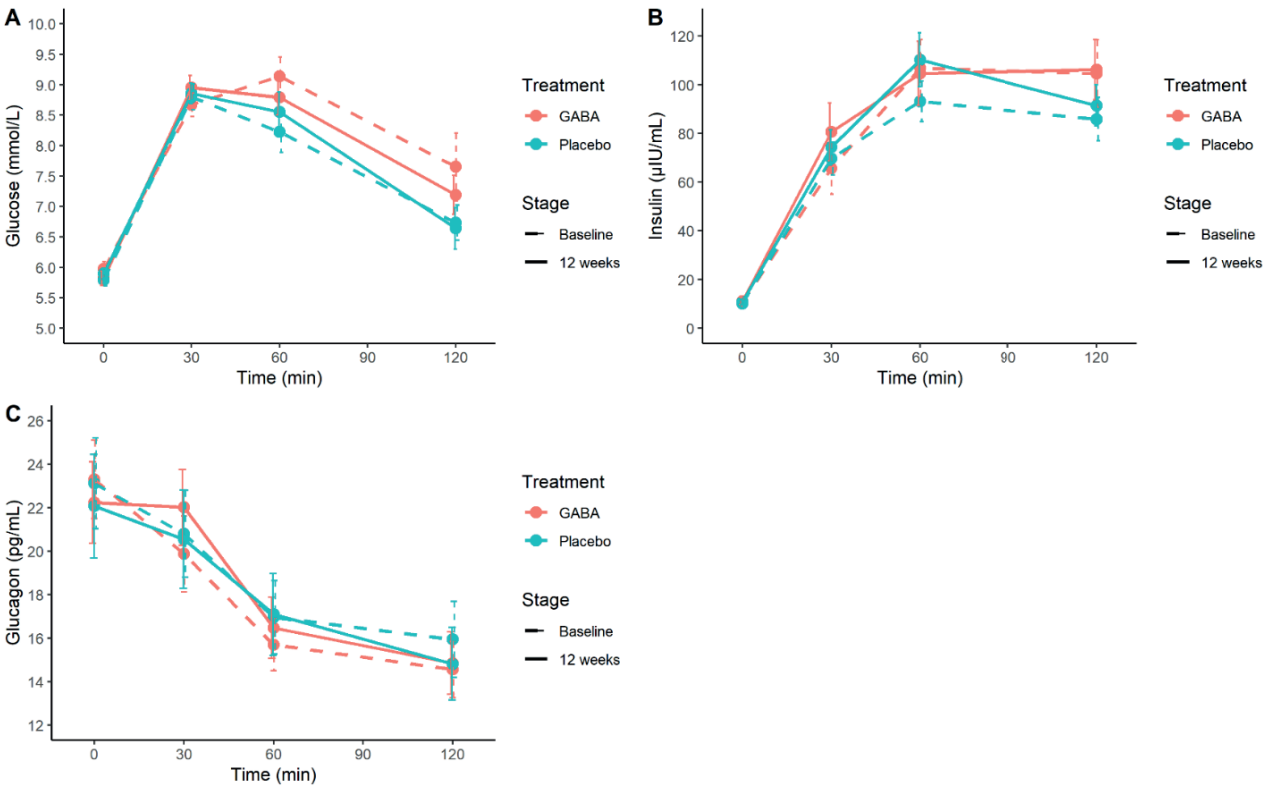


Figure 3: Post-intervention effects of GABA vs Placebo on analytes measured in plasma during an OGTT with 75 gr glucose on day 8 of the measurement periods (figure 1), A) glucose, B) insulin, and C) glucagon. *Blood was drawn at fasting, 30 minutes, 60 minutes, and 120 minutes after intake. Red lines represent the group allocated to the GABA intervention and blue lines represent the group allocated to the placebo. Filled lines represent the OGTT taken after the 3 month intervention, while dotted lines represent baseline plasma concentrations. Data are presented as mean with SEM error bars. Group differences in the postprandial response were analyzed using linear mixed models, and no significant differences were found. The insulin response was logarithmically transformed before the statistical analysis.*

Table 2: Effects of GABA on parameters related to glucose homeostasis.[1]

	Placebo, n_{max} = 25		**GABA, n_{max} = 24**		**Between-group change**	**P value**	**Adj. p value**[4]
	Baseline	Within-group change	Baseline	Within-group change			
Fasting glucose (mmol/L)	5.79 ± 0.47	0.10 ± 0.25	5.97 ± 0.59	-0.15 ± 0.36	-0.22 (-0.397, -0.045)	0.015	0.435
Fasting insulin (µIU/mL)	10.07 ± 4.98	0.66 ± 4.81	10.46 ± 7.16	0.70 ± 2.48	-0.01 (-0.108, 0.081)[2]	0.768	1.000
Fasting glucagon (pg/mL)	23.11 ± 10.26	-1.05 ± 6.36	23.30 ± 8.84	-1.06 ± 4.50	0.00 (-3.220, 3.221)	1.000	1.000
HbA1c (mmol/L)	37.96 ± 3.46	0.00 ± 1.38	39.00 ± 3.97	-0.62 ± 1.86	-0.47 (-1.389, 0.446)	0.306	1.000

Flash glucose monitoring[3]							
Mean glucose (mmol/L)	5.38 ± 0.57	-0.29 ± 0.46	5.69 ± 0.50	-0.45 ± 0.56	0.00 (-0.262, 0.264)	0.993	1.000
MAGE (mmol/L)	1.40 ± 0.32	-0.01 ± 0.26	1.67 ± 0.38	-0.06 ± 0.29	0.04 (-0.123, 0.202)	0.625	1.000
SD (mmol/L)	0.88 ± 0.20	-0.02 ± 0.15	1.05 ± 0.24	-0.06 ± 0.15	0.00 (-0.078, 0.097)	0.827	1.000
HOMA-IR	2.63 ± 1.34	0.20 ± 1.28	2.74 ± 1.74	0.11 ± 0.68	-0.03 (-0.126, 0.067)[2]	0.538	1.000

[1] Values are presented as mean ± SD. As between-group change, the estimates (95% CI) are presented derived from a one-way ANCOVA with treatment as the main effect and baseline value as a covariate.
[2] Log-transformed for normal distribution
[3] Flash glucose monitoring data of maximally 5 consecutive days was used. Participants with fewer than 3 days of glucose data were excluded. Placebo, n = 25; GABA, n = 22
[4] The adjusted p values were adjusted for false discovery rate using the Benjamini-Hochberg method

3.3. Post-intervention effects of GABA on exploratory secondary outcomes

Previously published literature presented indications of the potential effects of GABA on different cardiometabolic parameters and sleep quality. As part of the clinical trial, we, therefore, also explored the effects of GABA on blood pressure, lipid markers, and sleep quality. In addition, the fasting plasma GABA concentrations and its precursor glutamate were assessed before and after the intervention (~14 hours after taking the last capsule).

Blood pressure was measured both at the university by a trained researcher for a single blood pressure measurement and at home with an ambulatory blood pressure monitor for 24 hours. No significant treatment group differences were found in blood pressure (Table 3 and Figure 4). In addition, no significant differences were found in fasting plasma triglycerides, HDL- and LDL-cholesterol concentrations (table 3).

Sleep quality was assessed with a PSQI questionnaire. One of the participants in the placebo group had a PSQI score of 10 before the intervention and 1 after the intervention. With an average within-group change in PSQI score of -0.33±1.31, this change of 9 points is considered an outlier. Therefore, this participant's PSQI score was omitted from the analysis. Excluding the outlier changed the parameters of the model from $\beta = -0.51$ and $P = 0.21$ with the outlier to $\beta = -0.66$ and $P = 0.046$ without the outlier. Without the outlier, a significant improvement in self-reported sleep quality after the intervention in the GABA group as compared to placebo was observed (table 3). However, this was no longer a significant finding after correction for the false discovery rate.

The supplementation of GABA did not significantly elevate the fasting plasma GABA concentration compared to a placebo (table 3). Also, for glutamate, no differences were observed. Weight, waist circumference, and AST/ALT ratio remained stable during the intervention.

Table 3: Post-intervention effects of GABA on exploratory secondary outcomes[1]

	Placebo, n_{max} = 25		**GABA, n_{max} = 24**		**Between-group change**	**P value**	**Adj. p value[7]**
	Baseline	Within-group change	Baseline	Within-group change			
Blood pressure (mmHg)							
Systolic	130 ± 14.49	-1.15 ± 7.31	127 ± 11.83	0.03 ± 10.42	0.29 (-4.598, 5.181)	0.905	1.000
Diastolic	78 ± 9.27	1.77 ± 6.06	76 ± 9.91	1.17 ± 6.55	-0.71 (-4.345, 2.917)	0.694	1.000
24H-blood pressure (mmHg)[2]							
Systolic	126 ± 12.97	-0.07 ± 4.92	126 ± 8.28	-0.79 ± 6.53	-1.22 (-6.923, 4.486)[4]	0.668	1.000
Diastolic	75 ± 7.98	-0.09 ± 3.19	75 ± 7.09	0.07 ± 4.30	-0.07 (-4.448, 4.301)[4]	0.973	1.000

Blood pressure day (mmHg)[2]							
Systolic	130 ± 13.71	-0.27 ± 5.94	129 ± 8.79	-1.35 ± 7.62	-1.21 (-5.104, 2.684)	0.534	1.000
Diastolic	78 ± 8.40	-0.37 ± 3.27	77 ± 7.69	-0.22 ± 4.80	0.09 (-2.368, 2.538)	0.944	1.000
Blood pressure night (mmHg)[2]							
Systolic	116 ± 12.32	0.56 ± 5.38	115 ± 8.13	0.78 ± 6.56	0.04 (-3.251, 3.322)	0.983	1.000
Diastolic	66 ± 7.82	0.93 ± 4.67	67 ± 6.31	0.88 ± 5.17	0.02 (-2.871, 2.916)	0.987	1.000
Triglycerides (mmol/L)	1.38 ± 0.59	0.16 ± 0.38	1.58 ± 0.55	0.02 ± 0.39	-0.13 (-0.358, 0.101)[3]	0.266	1.000
HDL cholesterol (mmol/L)	1.33 ± 0.29	-0.05 ± 0.09	1.31 ± 0.29	-0.03 ± 0.12	0.02 (-0.043, 0.078)	0.560	1.000
LDL cholesterol (mmol/L)	3.14 ± 0.79	0.04 ± 0.46	3.26 ± 1.02	-0.09 ± 0.39	-0.10 (-0.327, 0.122)	0.363	1.000
Adiponectin (µg/ml)	7.28 ± 4.32	-0.40 ± 1.60	8.79 ± 5.04	0.04 ± 1.16	0.51 (-0.312, 1.330)	0.218	1.000
Pittsburgh Sleep Quality Index[5]	4.17 ± 2.82	-0.33 ± 1.31	3.45 ± 2.17	-0.71 ± 1.63	-0.66 (-1.301, -0.012)	0.046	0.667

GABA (ng/mL)[6]	**20.08 ± 3.45**	**-0.22 ± 2.04**	**18.94 ± 3.76**	**0.86 ± 2.15**	**0.68 (-0.329, 1.690)**	**0.181**	**1.000**
Glutamate (ng/mL)	3441 ± 1894	406 ± 1011	4154 ± 2683	-42 ± 1568	-251 (-938, 435)	0.465	1.000
Weight (kg)	90.98 ± 10.11	0.35 ± 1.43	89.13 ± 12.11	0.46 ± 1.55	0.17 (-0.677, 1.022)	0.685	1.000
Waist circumference (mm)	1048 ± 73.32	0.08 ± 25.81	1044 ± 82.38	4.75 ± 28.46	4.18 (-10.896, 19.246)	0.580	1.000
AST/ALT (IU/L)	0.93 ± 0.2	0.01 ± 0.16	0.92 ± 0.24	-0.01 ± 0.17	-0.03 (-0.114, 0.061)	0.542	1.000

[1] Values are presented as mean ± SD. As between-group change, the estimates (95% CI) are presented, derived from a one-way ANCOVA with treatment as the main effect and baseline value as a covariate, unless stated otherwise.
[2] Ambulatory 24-hour blood pressure measurement on day -2 (figure 1), an average per person was calculated. Averages are also split into day (09:00 to 21:00) and night (01:00 to 6:00). Participants with less than 70% of the data available were excluded. Placebo, n = 24; GABA, n = 20.
[3] Log-transformed for normal distribution.
[4] As between-group change, the estimates (95% CI) are presented derived from linear mixed models with treatment and time as fixed effects, participant identifier as random effect, and pre-intervention values as a covariate.
[5] One participant was excluded from the analysis since it deviated more than 3 standard deviations from the residuals. GABA, n=24; placebo, n=24.
[6] One participant was excluded as we were unable to measure GABA, possibly due to interference. GABA, n=23; placebo, n=24.
[7] The adjusted p values were adjusted for false discovery rate using the Benjamini-Hochberg method

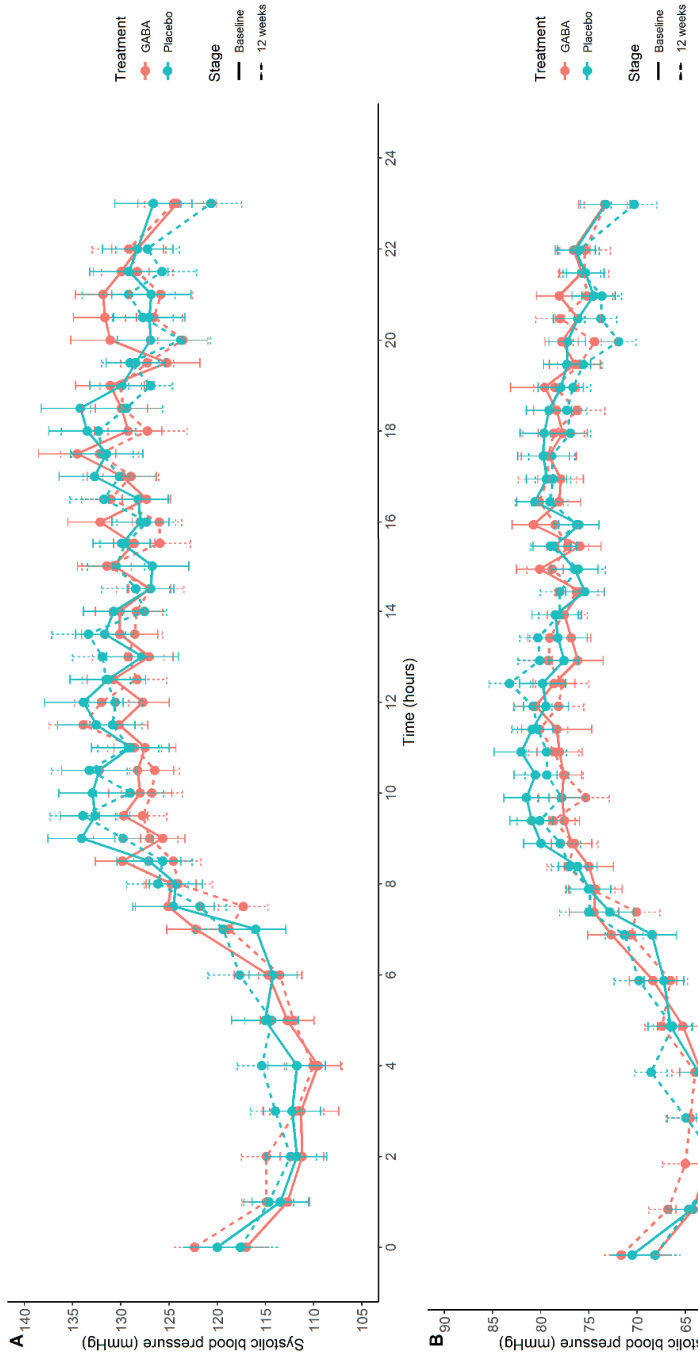


Figure 4: 24-hour ambulatory blood pressure measurements on day -2 and 97 (figure 1) of the measurement period, showing A) systolic blood pressure measurements and B) diastolic blood pressure measurements over a period of 24 hours. *Red lines represent the group allocated to the GABA intervention and blue lines represent the group allocated to the placebo. Filled lines represent the measurements taken after the 95 days intervention, while dotted lines represent 24-hour blood pressure before the intervention. Data are presented as mean with SEM error bars. Group differences in 24-hour blood pressure were analyzed using linear mixed models, and no significant differences were found.*

3.4. Acute effects of GABA

The acute effects of GABA were assessed at the start of the treatment period. Postprandial glucose levels were evaluated with flash glucose monitoring during an OGTT, reflecting interstitial glucose levels rather than blood glucose levels. Compared to a placebo, the glucose response was not different after the intake of a single GABA capsule (0.10 mmol/L; 95% CI: -0.361, 0.552; P: 0.678) (Figure 5). Ambulatory blood pressure was measured for 10 hours, and GABA or placebo capsules were taken at 9:00, 13:00, and 18:00 (Figure 6). No significant acute effects of GABA on blood pressure were found (-5.6 mmHg; 95% CI: -13.1, 2.0; P: 0.146).

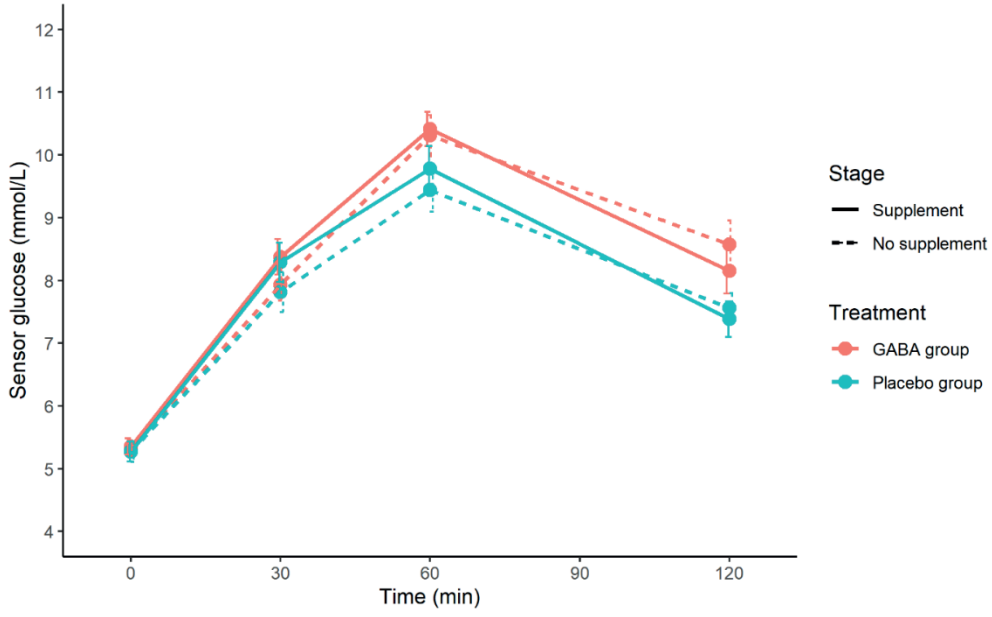


Figure 5: Acute effects on interstitial glucose as measured during a 75gr OGTT with flash glucose monitoring on days -3 and 1 at the start of the intervention. *Red lines represent the group allocated to the GABA intervention and blue lines represent the group allocated to the placebo. Filled lines represent the measurements taken when either a GABA or placebo capsule was taken together with the glucose drink (day 1 at the start of the intervention, figure 1), while dotted lines represent the pre-intervention glucose response when no capsules were taken yet (day -3 before the intervention, figure 1). Data are presented as mean with SEM error bars. Group differences in the postprandial response were analyzed using linear mixed models, and no significant differences were found.*

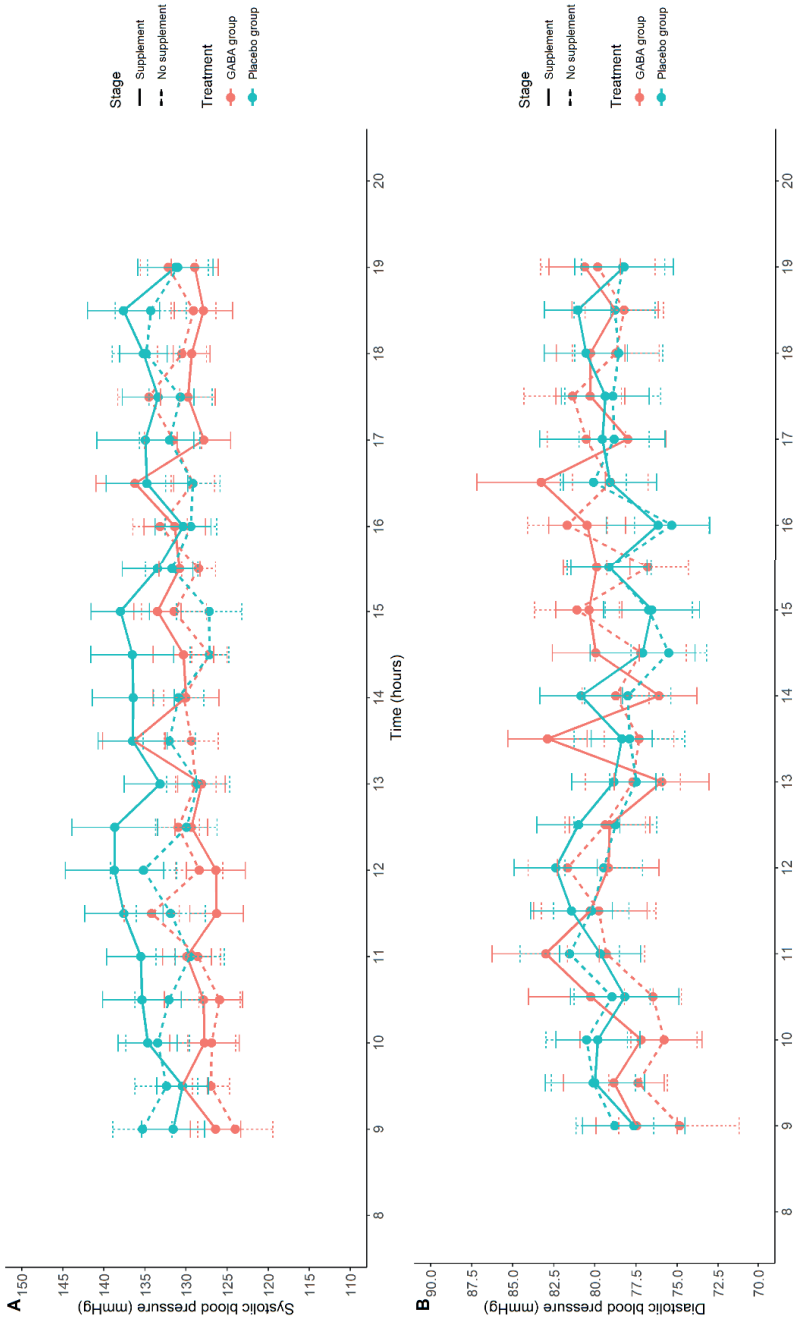


Figure 6: Acute effects of GABA recorded by 10-hour ambulatory blood pressure measurements on days -2 and 2 (see figure 1), showing A) systolic blood pressure measurements and B) diastolic blood pressure measurements over a period of 10 hours. *Red lines represent the group allocated to the GABA intervention and blue lines represent the group allocated to the placebo. Filled lines represent the measurements taken when either GABA or placebo capsules were taken (day 2, figure 1), while dotted lines represent 10-hour blood pressure when no capsules were taken (day -2, figure 1). On day 2 capsules were taken at 9:00, 13:00, and 18:00. Data are presented as mean with SEM error bars. Group differences in 10-hour blood pressure were analyzed using linear mixed models, and no significant differences were found.*

3.5. Testing for interaction with baseline value

The presence of an interaction between the treatment and the baseline value was tested for each parameter. A significant interaction with baseline value was found for post-intervention systolic blood pressure, PSQI score, and HDL cholesterol concentration. Therefore, for these outcomes, the baseline value influences the relationship between the treatment and the post-intervention value. The regression parameters for the interaction effect of treatment with the respective baseline value are as follows: -0.37 mmHg; 95% CI: -0.718, -0.018; P: 0.040 for blood pressure, -0.30; 95% CI: -0.554, -0.042; P: 0.023 for PSQI score and 0.22 mmol/L; 95% CI: 0.012, 0.420; P: 0.038 for HDL cholesterol.

These findings were further investigated and visualized with Johnson-Neyman plots, plotting the estimated effect size and confidence interval against the baseline value (Figure 7). Only for the PSQI score this interaction persisted after correcting for multiple testing. The effect of the GABA treatment on post-intervention PSQI score is significant, above a baseline PSQI score of 3.75.

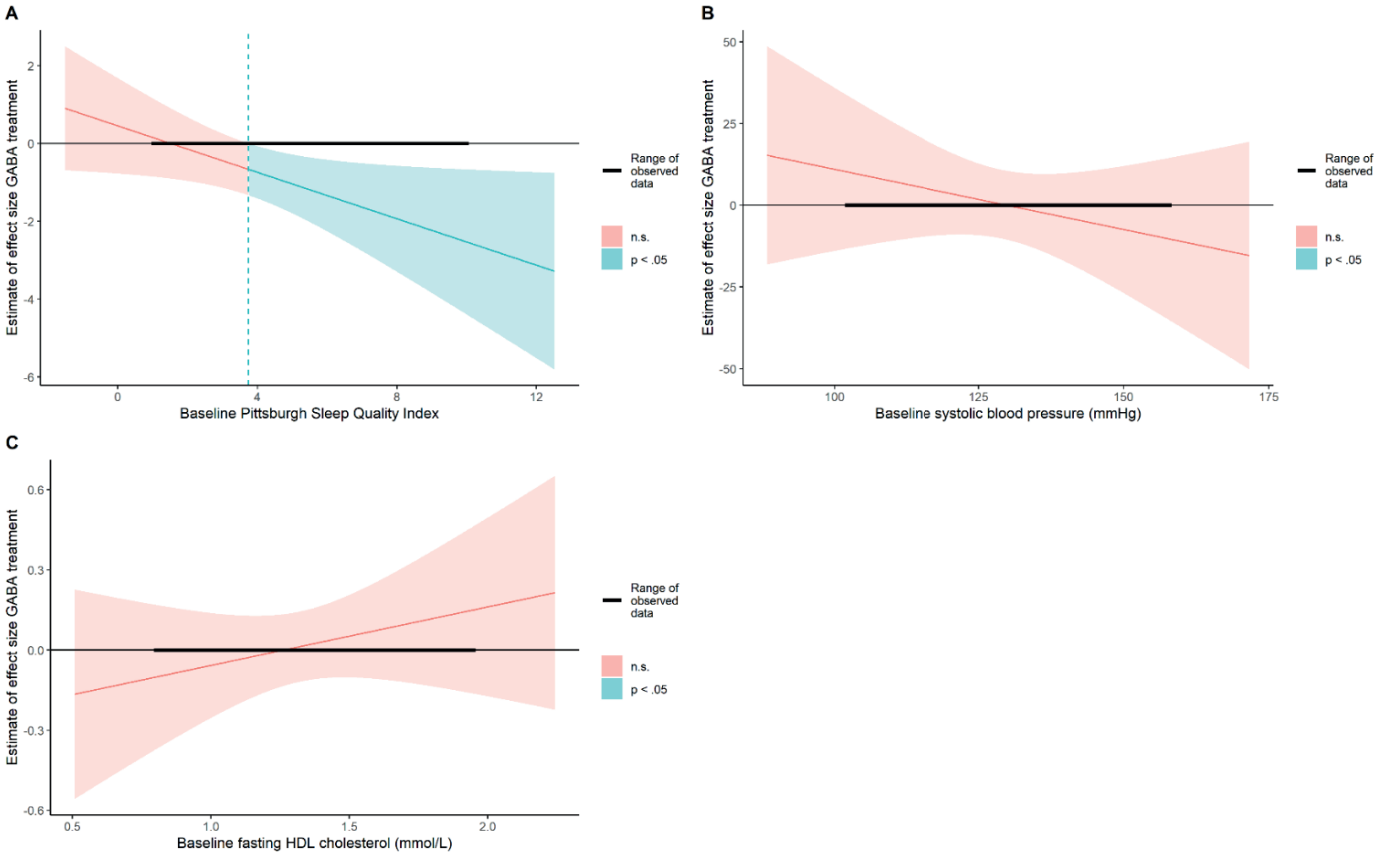


Figure 7: Johnson-Neyman plots showing the estimated effect size of the GABA treatment and confidence interval plotted against the pre-intervention (baseline value) of the A) Pittsburgh Sleep Quality Index, B) systolic blood pressure and C) HDL cholesterol concentration. *The green area shows the range of the pre-intervention value in which the GABA treatment has a significant (P<0.05) effect as compared to the placebo group. Correction for multiple testing was applied.*

3.6. Adverse events

No severe adverse events were reported. In the placebo group, 32% of the participants reported mild adverse events, while in the GABA group this was 44% (Table 4). Headache, tingling feeling, light-headedness, and sensitive arms and legs were solely reported in the GABA group.

Table 4: reported adverse events during intervention[1]

	GABA		Placebo	
Adverse event	n	%	n	%
Gastrointestinal complaints	3	12	2	8
Tiredness	2	8	2	8
Headache	2	8	0	0
Cystitis	1	4	1	4
Tingling feeling	1	4	0	0
Lightheaded after exercise	1	4	0	0
Sensitive arms and legs	1	4	0	0
Insomnia	0	0	1	4
Dizziness	0	0	2	8
Total	**11**	**44**	**8**	**32**

[1] n, number of participants that reported the adverse event; %, percentage of total included participants.

4. Discussion

In this double-blind, randomized, placebo-controlled intervention study with adults at risk of developing type 2 diabetes, we assessed whether 95 days of 1500 mg GABA supplementation per day could improve glucose homeostasis, as reflected primarily by the glucose response following an OGTT. Next to the primary outcome, we explored the effects of GABA on a broad spectrum of secondary outcomes for cardiometabolic health and sleep quality. GABA supplementation did not change the postprandial glucose, insulin, and glucagon response. Although we obtained some indication that GABA supplementation could reduce the fasting blood glucose concentration and improve the sleep quality of the participants, these results did not remain significant after correction for the false discovery rate. Therefore, they can only be interpreted as explorative findings. We observed no effects of GABA supplementation on any of the other secondary outcomes. Additionally, it should be noted that the basal (fasting) plasma levels of GABA did not increase after 95 days of supplementation, suggesting that there is no accumulation at this dosage regimen.

These findings are contrary to our hypothesis, based on previous studies that suggested GABA would have a beneficial effect on the development of diabetes in individuals at risk. However, these earlier studies are mostly done with rodent models of diabetes or hypertension. Only a few human studies allow some direct comparisons to our intervention trial regarding dosing, duration, and measurement of effects. The study by Li et al. [16] looked primarily at the plasma kinetics of GABA in healthy participants after a single dose of 2 grams and a repeated dose (3 times daily, 2 grams for seven days) (16). After intake of GABA in both the single dose and repeated dose period, their results show an increase in insulin concentration under both fasting and post-meal conditions. Even with a single GABA dose in the morning, there was a significant effect of GABA on post-meal insulin during the day (after lunch and dinner). Glucagon was only raised under fasting conditions, 4 hours after GABA intake. However, no effect on glucose levels was observed in these healthy individuals. The authors did observe a significant decrease in glycated albumin (a short-term measure of glycemic control) after seven days of repeated dosing. After three months, we did not observe any such improvement in HbA1c (a marker of more long-term glycemic control). It is important to note that the dosage employed in that study is not only significantly higher than that of our current investigation, but it also surpasses the commonly administered dosage of GABA supplements and that which can be obtained from a regular diet.

In another study, a single dose of GABA was administered in different doses to patients with type 1 diabetes [29]. Under hypoglycemic conditions, a dose of 600 mg GABA increased glucagon, epinephrine, growth hormone, and cortisol plasma concentrations, but there were no effects in a normoglycemic situation. Although we did not evaluate the acute effects of a single dose of GABA on postprandial glucagon and insulin

response, we found no alterations in fasting glucagon and insulin plasma levels, or in the postprandial glucagon and insulin response after 95 days of GABA supplementation. In line with the results of other studies, we also showed that GABA intake does not acutely change the postprandial glucose response. This suggests that any acute changes in hormone response after GABA intake do not lead to a change in glucose response in this population, neither acutely nor after 95 days of supplementation. It remains interesting to study the acute effects of GABA on insulin and glucagon response in individuals with (pre-)diabetes in future studies.

Studies performed in rodent models have suggested that GABA is able to restore beta-cell function and mass and reduce low-grade inflammation. Untereiner et al. [30] used a diet-induced obesity mouse model for prediabetes and administered GABA via drinking water as a 6 mg/mL solution [30]. Interestingly, they found no effects of GABA (administered in drinking water for 10 weeks) in the mice that developed pre-diabetes-like symptoms. Remarkably, they found an increased beta-cell mass, improved glucose tolerance, and increased insulin secretion in healthy control mice after GABA administration. Still, they observed no changes in glucagon secretion or insulin sensitivity.

Untereiner et al. [30] hypothesized that GABA does not impact glucose tolerance in the prediabetic model since hyperinsulinemia and expansion of beta-cell mass already occur during the development of obesity to compensate for insulin resistance. Any potential effect of GABA on increasing beta-cell mass and insulin secretion would only be pertinent in a diseased diabetic state, where there has been a loss of beta-cell mass and a decrease of insulin secretion. This theory would be in line with the lack of effect of GABA on glucose tolerance that we observed in the current study. Tian et al. [15] did find an improvement in glucose tolerance and insulin sensitivity in mice after 20 weeks of 2 mg/mL GABA in drinking water together with a high-fat diet (15). However, they did not assess the effect on beta-cell mass, and GABA treatment also reduced weight gain relative to the control, which was not the case in the study by Untereiner et al. [30].

Although the possibility of a chance finding cannot be ruled out, the decrease in fasting plasma glucose by 0.22 mmol/L (95% CI: 0.397, 0.045) following 95 days of GABA intake would be of interest to explore further. The observed decrease occurred independently of the fasting glucose concentration at the start of the intervention, and this effect size is of the same order of magnitude as found in other studies investigating bioactive food components or lifestyle interventions [31–33]. For example, in a study performed in our institute, Schutte et al. [34] found that a 12-week, 25% energy restriction trial that successfully induced weight loss led to a reduction in fasting glucose concentration of 0.3 mmol/L in a comparable population (35). The fasting glucose concentration is normally tightly controlled in a range from 3.9 to 6.9 mmol/L and even small changes can substantially impact health outcomes. For prediabetes, estimates suggest that a 0.5 mmol/L increase in fasting glucose concentration would

be associated with a 2- to 3-fold increased risk of progression to diabetes [35–37]. In this regard, the observed reduction in fasting glucose could be considered of interest for further research. At the same time, effects seen with successful lifestyle interventions are usually broader in terms of their health outcomes. Moreover, the current dosing regimen of 500 mg, three times daily, in combination with this relatively small effect, would not be clinically or nutritionally attractive and is likely to result in low compliance.

We also investigated the potential effects of GABA on self-reported sleep quality, the reason for this being that GABA supplements are widely advocated for this purpose. Although this secondary outcome measure of our study seems to point at such an effect, it should be interpreted at most as indicative of a possible effect since the finding was no longer significant after correction for the false discovery rate. In addition, an outlier was removed from the data analysis. With an average PSQI score below 5, the included participants can be characterized as good sleepers [22]. The effect of GABA supplementation might be most relevant in poor sleepers, especially since we observed an effect size that is dependent on pre-existing self-reported sleep quality. Studies investigating the effects of GABA on sleep quality in humans are scarce and of short duration [38,39]. Therefore, substantiation for improving sleep quality by GABA remains very limited, and further research focusing on sleep quality in poor sleepers is required. Also, for blood pressure, a possible effect might be dependent on pre-existing blood pressure. Although this interaction did not hold up after a correction for multiple testing, this warrants specific research in hypertensive individuals. Previous studies also found no effect of GABA under normotensive conditions, which is in line with our results [40,41].

We showed that after 95 days of supplementation, fasting GABA plasma concentrations were not elevated. This is in line with our previous kinetic study (17), in which we observed that plasma levels steeply rise after oral GABA intake but return to normal in less than 5 hours. Apparently, GABA does not accumulate in plasma; therefore, plasma GABA levels are not a good marker for GABA intake. Another study also showed no accumulation of GABA in plasma after administration of 2 grams three times a day for one week [16]. Both studies indicate that GABA is rapidly cleared from the blood, most likely as a consequence of metabolism or distribution to tissues/organs like the kidney cortex, liver, pineal gland, and pituitary gland [42]. At the same time, positive health effects of other substances, such as green tea catechins, are observed to not depend on prolonged elevated plasma levels [33,43,44]. Further research should focus on identifying biomarkers of GABA intake, like metabolites or conjugates of GABA, to be able to further investigate any health effects of a GABA-rich diet.

Strengths and limitations

Major strengths of this study are its double-blind, randomized, placebo-controlled design and long duration. Next to this, compliance was high, and the drop-out rate was minimal. However, there are also some limitations of the study that need to be acknowledged. Depending on the comparison, the dosage regimen of 3 daily doses of 500 mg GABA is relatively low compared to the doses given to rodents in previous studies and relatively high compared to the estimated dietary GABA intake [45]. Higher doses of GABA could be administered orally since no severe adverse events of GABA were found up to doses of 18 grams [46]. However, it would be impossible to reach a daily intake of this magnitude through dietary intake. This leads us to believe that there is little chance that, with the current dose, we will have missed important effects that GABA could have through dietary intake, at least in this population. In addition, the study was conducted in individuals with a relatively early and still mild stage of prediabetes. This was motivated, on the one hand, by consideration of the status of GABA as a nutritional ingredient and, on the other hand, to circumvent medication use as a possible confounding factor.

The methods we used also have some limitations that should be considered when interpreting the results. We standardized the diet before the OGTT to avoid an increase in variability due to diet and activities in the preceding days [47]. Therefore, standardizing the diet before the OGTT could have reduced the within-person variability. Furthermore, recording a food diary and following a specific diet changes behavior and could affect glucose homeostasis. The glucose monitor the participants used was also not blinded, so they were aware of their glucose levels.

Conclusion

In conclusion, the present study shows no effect of 95 days of oral GABA supplementation on the postprandial glucose response in participants at risk of developing type 2 diabetes. Although we find some indication for a reduction in the fasting plasma glucose concentration, the present results do not provide sufficient support to recommend GABA-rich products or GABA supplements to individuals at risk of developing (pre-)diabetes. Further research should clarify the mechanism of action behind the potential health effects of GABA. This is supported by some of the explorative secondary outcomes on sleep quality and hypertension.

References

[1] J.C. Gerber, T.A. Hare, GABA in peripheral tissues: Presence and actions in endocrine pancreatic function, Brain Res Bull. 5 (1980) 341–346. https://doi.org/10.1016/0361-9230(80)90055-6.

[2] S. Sen, S. Roy, G. Bandyopadhyay, B. Scott, D. Xiao, S. Ramadoss, S.K. Mahata, G. Chaudhuri, γ-Aminobutyric Acid Is Synthesized and Released by the Endothelium: Potential Implications, Circ Res. 119 (2016) 621–634. https://doi.org/10.1161/CIRCRESAHA.116.308645.

[3] M. Watanabe, K. Maemura, K. Kanbara, T. Tamayama, H. Hayasaki, GABA and GABA Receptors in the Central Nervous System and Other Organs, Int Rev Cytol. 213 (2002) 1–47. https://doi.org/10.1016/S0074-7696(02)13011-7.

[4] A.K. Bhandage, A. Barragan, GABAergic signaling by cells of the immune system: more the rule than the exception, Cellular and Molecular Life Sciences 2021 78:15. 78 (2021) 5667–5679. https://doi.org/10.1007/S00018-021-03881-Z.

[5] R. Mazzoli, E. Pessione, The Neuro-endocrinological Role of Microbial Glutamate and GABA Signaling, Front Microbiol. 7 (2016) 1934. https://doi.org/10.3389/fmicb.2016.01934.

[6] T. Toyoizumi, S. Ohba, K. suke Fujii, A. Ikegaya, H. Matsuura, T. Nakajima, Differential GABA concentration gradients are present in the edible parts of greenhouse melon (Cucumis melo L.) during all four seasonal croppings, Biosci Biotechnol Biochem. 83 (2019) 330–338. https://doi.org/10.1080/09168451.2018.1537774.

[7] B. Redruello, A. Szwengiel, V. Ladero, B. del Rio, M.A. Alvarez, Identification of technological/metabolic/environmental profiles of cheeses with high GABA contents, LWT. (2020) 109603. https://doi.org/10.1016/J.LWT.2020.109603.

[8] T. Saito, C. Matsukura, M. Sugiyama, A. Watahiki, I. Ohshima, Y. Iijima, C. Konishi, T. Fujii, S. Inai, N. Fukuda, S. Nishimura, H. Ezura, Screening for γ-aminobutyric acid (GABA)-rich tomato varieties, Journal of the Japanese Society for Horticultural Science. 77 (2008) 242–250. https://doi.org/10.2503/jjshs1.77.242.

[9] S. Nonaka, C. Arai, M. Takayama, C. Matsukura, H. Ezura, Efficient increase of ɣ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis, Sci Rep. 7 (2017) 1–14. https://doi.org/10.1038/s41598-017-06400-y.

[10] D.W. Hagan, S.M. Ferreira, G.J. Santos, E.A. Phelps, The role of GABA in islet function, Front Endocrinol (Lausanne). 13 (2022). https://doi.org/10.3389/FENDO.2022.972115.

[11] D. Menegaz, D.W. Hagan, J. Almaça, C. Cianciaruso, R. Rodriguez-Diaz, J. Molina, R.M. Dolan, M.W. Becker, P.C. Schwalie, R. Nano, F. Lebreton, C. Kang, R. Sah, H.Y. Gaisano, P.-O. Berggren, S. Baekkeskov, A. Caicedo, E.A. Phelps, Mechanism and

effects of pulsatile GABA secretion from cytosolic pools in the human beta cell, Nat Metab. 1 (2019) 1110–1126. https://doi.org/10.1038/s42255-019-0135-7.

[12] I. Purwana, J. Zheng, X. Li, M. Deurloo, D.O. Son, Z. Zhang, C. Liang, E. Shen, A. Tadkase, Z.-P. Feng, Y. Li, C. Hasilo, S. Paraskevas, R. Bortell, D.L. Greiner, M. Atkinson, G.J. Prud'homme, Q. Wang, GABA promotes human β-cell proliferation and modulates glucose homeostasis, Diabetes. 63 (2014) 4197–4205. https://doi.org/10.2337/db14-0153.

[13] N. Soltani, H. Qiu, M. Aleksic, Y. Glinka, F. Zhao, R. Liu, Y. Li, N. Zhang, R. Chakrabarti, T. Ng, T. Jin, H. Zhang, W.-Y. Lu, Z.-P. Feng, G.J. Prud'homme, Q. Wang, GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes., Proc Natl Acad Sci U S A. 108 (2011) 11692–7. https://doi.org/10.1073/pnas.1102715108.

[14] S. Sohrabipour, M.R. Sharifi, A. Talebi, M. Sharifi, N. Soltani, GABA dramatically improves glucose tolerance in streptozotocin-induced diabetic rats fed with high-fat diet, Eur J Pharmacol. 826 (2018) 75–84. https://doi.org/10.1016/J.EJPHAR.2018.01.047.

[15] J. Tian, H.N. Dang, J. Yong, W.-S. Chui, M.P.G. Dizon, C.K.Y. Yaw, D.L. Kaufman, Oral Treatment with γ-Aminobutyric Acid Improves Glucose Tolerance and Insulin Sensitivity by Inhibiting Inflammation in High Fat Diet-Fed Mice, PLoS One. 6 (2011) e25338. https://doi.org/10.1371/journal.pone.0025338.

[16] J. Li, Z. Zhang, X. Liu, Y. Wang, F. Mao, J. Mao, X. Lu, D. Jiang, Y. Wan, J.-Y. Lv, G. Cao, J. Zhang, N. Zhao, M. Atkinson, D.L. Greiner, G.J. Prud'homme, Z. Jiao, Y. Li, Q. Wang, Study of GABA in Healthy Volunteers: Pharmacokinetics and Pharmacodynamics, Front Pharmacol. 6 (2015) 260. https://doi.org/10.3389/fphar.2015.00260.

[17] T.H. de Bie, M.G.J. Balvers, R.C.H. de Vos, R.F. Witkamp, M.A. Jongsma, The influence of a tomato food matrix on the bioavailability and plasma kinetics of oral gamma-aminobutyric acid (GABA) and its precursor glutamate in healthy men, Food Funct. (2022). https://doi.org/10.1039/D2FO01358D.

[18] F. Magkos, M.F. Hjorth, A. Astrup, Diet and exercise in the prevention and treatment of type 2 diabetes mellitus, Nature Reviews Endocrinology 2020 16:10. 16 (2020) 545–555. https://doi.org/10.1038/s41574-020-0381-5.

[19] L. Perreault, Q. Pan, K.J. Mather, K.E. Watson, R.F. Hamman, S.E. Kahn, Effect of regression from prediabetes to normal glucose regulation on long-term reduction in diabetes risk: results from the Diabetes Prevention Program Outcomes Study, The Lancet. 379 (2012) 2243–2251. https://doi.org/10.1016/S0140-6736(12)60525-X.

[20] T.H. de Bie, R.F. Witkamp, M.A. Jongsma, M.G.J. Balvers, Development and validation of a UPLC-MS/MS method for the simultaneous determination of gamma-aminobutyric acid and glutamic acid in human plasma, J Chromatogr B Analyt

Technol Biomed Life Sci. 1164 (2021) 122519. https://doi.org/10.1016/j.jchromb.2020.122519.

[21] F.J. Service, G.D. Molnar, J.W. Rosevear, E. Ackerman, L.C. Gatewood, W.F. Taylor, Mean Amplitude of Glycemic Excursions, a Measure of Diabetic Instability, Diabetes. 19 (1970) 644–655. https://doi.org/10.2337/DIAB.19.9.644.

[22] D.J. Buysse, C.F. Reynolds, T.H. Monk, S.R. Berman, D.J. Kupfer, The Pittsburgh sleep quality index: A new instrument for psychiatric practice and research, Psychiatry Res. 28 (1989) 193–213. https://doi.org/10.1016/0165-1781(89)90047-4.

[23] S.E. Park, O.-H. Kim, J.H. Kwak, K.-H. Lee, Y.-I. Kwon, K.H. Chung, J.H. Lee, Antihyperglycemic effect of short-term arginyl-fructose supplementation in subjects with prediabetes and newly diagnosed type 2 diabetes: randomized, double-blinded, placebo-controlled trial, Trials. 16 (2015) 521. https://doi.org/10.1186/s13063-015-1036-z.

[24] P.O. Johnson, L.C. Fay, The Johnson-Neyman technique, its theory and application, Psychometrika 1950 15:4. 15 (1950) 349–367. https://doi.org/10.1007/BF02288864.

[25] J.A. Long, interactions: Comprehensive, User-Friendly Toolkit for Probing Interactions, (2019).

[26] J. Esarey, J.L. Sumner, Marginal Effects in Interaction Models: Determining and Controlling the False Positive Rate, Comp Polit Stud. 51 (2018) 1144–1176. https://doi.org/10.1177/0010414017730080.

[27] Y. Benjamini, Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing, Journal of the Royal Statistical Society: Series B (Methodological). 57 (1995) 289–300. https://doi.org/10.1111/J.2517-6161.1995.TB02031.X.

[28] H. Wickham, ggplot2: Elegant Graphics for Data Analysis, Springer New York, New York, NY, 2009. https://doi.org/10.1007/978-0-387-98141-3.

[29] D. Espes, H. Liljebäck, H. Hill, A. Elksnis, J. Caballero-Corbalan, P.-O. Carlsson, GABA induces a hormonal counter-regulatory response in subjects with long-standing type 1 diabetes, BMJ Open Diabetes Res Care. 9 (2021) e002442. https://doi.org/10.1136/BMJDRC-2021-002442.

[30] A. Untereiner, S. Abdo, A. Bhattacharjee, H. Gohil, F. Pourasgari, N. Ibeh, M. Lai, B. Batchuluun, A. Wong, N. Khuu, Y. Liu, D. Al Rijjal, N. Winegarden, C. Virtanen, B.A. Orser, O. Cabrera, G. Varga, J. Rocheleau, F.F. Dai, M.B. Wheeler, GABA promotes b-cell proliferation, but does not overcome impaired glucose homeostasis associated with diet-induced obesity, The FASEB Journal. (2018). https://doi.org/10.1096/fj.201801397R.

[31] Diabetes Prevention Program Research Group, Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin, Https://Doi.Org/10.1056/NEJMoa012512. 346 (2002) 393–403. https://doi.org/10.1056/NEJMOA012512.

[32] X.J. Yang, S.F. Zou, Y. Xu, Y. Li, S.S. Yang, The influence of intensive lifestyle intervention on patients with isolated impaired fasting glucose: a meta-analysis, J Adv Nurs. 72 (2016) 2587–2597. https://doi.org/10.1111/JAN.13015.

[33] X.X. Zheng, Y.L. Xu, S.H. Li, R. Hui, Y.J. Wu, X.H. Huang, Effects of green tea catechins with or without caffeine on glycemic control in adults: a meta-analysis of randomized controlled trials, Am J Clin Nutr. 97 (2013) 750–762. https://doi.org/10.3945/AJCN.111.032573.

[34] S. Schutte, D. Esser, E. Siebelink, C.J.R. Michielsen, M. Daanje, J.C. Matualatupauw, H.C. Boshuizen, M. Mensink, L.A. Afman, T.W.B.F.S. team, S. Schutte, D. Esser, E. Siebelink, H. Fick, M.M. Grootte Bromhaar, Y. Wang, S.E.M. de Bruijn, M. Mars, J. Meijerink, M. Mensink, L.A. Afman, E.J.M. Feskens, M. Müller, Diverging metabolic effects of 2 energy-restricted diets differing in nutrient quality: a 12-week randomized controlled trial in subjects with abdominal obesity, Am J Clin Nutr. 116 (2022) 132–150. https://doi.org/10.1093/AJCN/NQAC025.

[35] S. Soulimane, D. Simon, J. Shaw, D. Witte, P. Zimmet, S. Vol, K. Borch-Johnsen, D. Magliano, D. Vistisen, B. Balkau, HbA1c, fasting plasma glucose and the prediction of diabetes: Inter99, AusDiab and D.E.S.I.R., Diabetes Res Clin Pract. 96 (2012) 392–399. https://doi.org/10.1016/J.DIABRES.2011.06.003.

[36] M. Tohidi, A. Baghbani-Oskouei, N.S. Ahanchi, F. Azizi, F. Hadaegh, Fasting plasma glucose is a stronger predictor of diabetes than triglyceride–glucose index, triglycerides/high-density lipoprotein cholesterol, and homeostasis model assessment of insulin resistance: Tehran Lipid and Glucose Study, Acta Diabetol. 55 (2018) 1067–1074. https://doi.org/10.1007/S00592-018-1195-Y/FIGURES/2.

[37] Y. Heianza, S. Hara, Y. Arase, K. Saito, K. Fujiwara, H. Tsuji, S. Kodama, S.D. Hsieh, Y. Mori, H. Shimano, N. Yamada, K. Kosaka, H. Sone, HbA1c 5·7–6·4% and impaired fasting plasma glucose for diagnosis of prediabetes and risk of progression to diabetes in Japan (TOPICS 3): a longitudinal cohort study, The Lancet. 378 (2011) 147–155. https://doi.org/10.1016/S0140-6736(11)60472-8.

[38] J.I. Byun, Y.Y. Shin, S.E. Chung, W.C. Shin, Safety and Efficacy of Gamma-Aminobutyric Acid from Fermented Rice Germ in Patients with Insomnia Symptoms: A Randomized, Double-Blind Trial, Journal of Clinical Neurology. 14 (2018) 291–295. https://doi.org/10.3988/JCN.2018.14.3.291.

[39] A. Yamatsu, Y. Yamashita, I. Maru, J. Yang, J. Tatsuzaki, M. Kim, The Improvement of Sleep by Oral Intake of GABA and Apocynum venetum Leaf Extract, J Nutr Sci Vitaminol (Tokyo). 61 (2015) 182–187. https://doi.org/10.3177/JNSV.61.182.

[40] H. Tanaka, K. Watanabe, M. Ma, M. Hirayama, T. Kobayashi, H. Oyama, Y. Sakaguchi, M. Kanda, M. Kodama, Y. Aizawa, The Effects of gamma-Aminobutyric Acid, Vinegar, and Dried Bonito on Blood Pressure in Normotensive and Mildly or Moderately Hypertensive Volunteers., J Clin Biochem Nutr. 45 (2009) 93–100. https://doi.org/10.3164/jcbn.09-04.

[41] H. Takahashi, M. Sumi, F. Koshino, Effect of gamma-aminobutyric acid (GABA) on normotensive or hypertensive rats and men., Jpn J Physiol. 11 (1961) 89–95.

[42] E. Kuroda, M. Watanabe, T. Tamayama, M. Shimada, Autoradiographic distribution of radioactivity from 14C-GABA in the mouse, Microsc Res Tech. 48 (2000) 116–126. https://doi.org/10.1002/(SICI)1097-0029(20000115)48:2<116::AID-JEMT7>3.0.CO;2-P.

[43] J.S. Wang, H. Luo, P. Wang, L. Tang, J. Yu, T. Huang, S. Cox, W. Gao, Validation of green tea polyphenol biomarkers in a phase II human intervention trial, Food and Chemical Toxicology. 46 (2008) 232–240. https://doi.org/10.1016/J.FCT.2007.08.007.

[44] Z.Y. Cai, X.M. Li, J.P. Liang, L.P. Xiang, K.R. Wang, Y.L. Shi, R. Yang, M. Shi, J.H. Ye, J.L. Lu, X.Q. Zheng, Y.R. Liang, Bioavailability of Tea Catechins and Its Improvement, Molecules 2018, Vol. 23, Page 2346. 23 (2018) 2346. https://doi.org/10.3390/MOLECULES23092346.

[45] M. Hermanussen, U. Gonder, C. Jakobs, D. Stegemann, G. Hoffmann, Patterns of free amino acids in German convenience food products: marked mismatch between label information and composition, European Journal of Clinical Nutrition 2010 64:1. 64 (2009) 88–98. https://doi.org/10.1038/ejcn.2009.116.

[46] A.D. and A.M. F. Cavagnini, C. Invitti, M. Pinto, C. Maraschini, A. Di Landro, Effect of acute and repeated administration of gamma aminobutyric acid (GABA) on growth hormone and prolactin secretion in man, Eur J Endocrinol. 93 (1980) 149–154.

[47] S. Numao, H. Kawano, N. Endo, Y. Yamada, M. Konishi, M. Takahashi, S. Sakamoto, Effects of a single bout of aerobic exercise on short-term low-carbohydrate/high-fat intake-induced postprandial glucose metabolism during an oral glucose tolerance test, Metabolism. 62 (2013) 1406–1415. https://doi.org/10.1016/J.METABOL.2013.05.005.

Chapter 6

General discussion

The aim of the current thesis was to investigate GABA as a bioactive plant component, commonly present in the human diet, and its potential to influence human metabolic health. To this end, the variation in GABA content between different potato and tomato genotypes was investigated and the effects of domestic cooking methods were studied. Next, a UPLC-MS/MS based method to quantify GABA in human plasma was developed and validated. This method was applied to study the relative bioavailability of GABA from a tomato food matrix as compared to GABA dissolved in water. Finally, the potential health effects of GABA supplementation in individuals with prediabetes were explored. Together, this thesis showed that, although the diet can be a good source of GABA and can lead to increased GABA exposure, our studies with GABA supplementation suggest that increased GABA intake has only modest effects in a population with prediabetes.

Summary of the main findings

The naturally occurring GABA content in a collection of different tomato and potato genotypes varied from 72 to 994 µg per gram fresh weight (FW) in tomato fruits and from 68 to 759 µg/g FW in potato tubers (**chapter 2**). In both crops, the GABA concentration was not correlated to that of its precursor glutamic acid. The highest GABA concentrations were found in tomato fruits from cv. 'Madara' and in potato tubers from cv. 'Riviera'. The Madara tomato fruits contained about 4 times as much GABA as the average fruits and the Riviera potato tubers contained about 2 times as much GABA as the average tuber. Considering that GABA content was found to be strongly reproducible across different harvest years with similar growth conditions, a high GABA content seems to be genetically determined and can be selected via plant breeding. Although losses in GABA content were observed during domestic cooking, the GABA content was not significantly reduced after baking and pan-frying potatoes and baking and boiling tomatoes. Overall, potatoes and tomatoes could contribute to dietary GABA intake as sources of GABA, if high-yielding cultivars are made available to the consumer.

No standard method to quantify base GABA plasma levels is available and validation is required for application in clinical trials. Therefore, an analytical method to be able to quantify base levels of GABA and its precursor glutamic acid in human plasma was developed and validated in **chapter 3**. The method was applied in the human studies described in chapters 4 and 5. The sensitivity of the method was sufficient to determine base fasting plasma concentrations of GABA and glutamic acid. Within- and between-day accuracy and precision were <10% in quality control samples at low, medium, and high concentrations for both GABA and glutamic acid. In addition, GABA and glutamic

acid were found to be stable in plasma after freeze-thaw cycles and up to 12 months of storage. The validated method was used to determine GABA concentrations in plasma from healthy volunteers, which showed concentrations that were in line with values that were previously reported by others in the literature.

In **chapter 4** results are presented from a randomized 4-way cross-over study, which showed that GABA is similarly bioavailable from a liter of pureed tomatoes as from a liter of GABA solution in water with the same GABA concentration. Kinetic parameters like the maximum concentration, the time to peak and the area under the curve, were comparable between the two conditions. While there were substantial interindividual differences, the fasting plasma GABA concentrations stayed relatively stable (5.8 CV%) within a person over the course of 4 weeks. In addition, intake of glutamic acid did not influence GABA plasma concentration, nor the other way around.

In **chapter 5** a randomized, placebo-controlled, double-blind, parallel-arm trial is described. In the trial, the post-intervention effects of 3 months GABA supplementation (1500 mg/d) on individuals with prediabetes were investigated. GABA supplementation did not improve the postprandial glucose response. The effect of GABA on several secondary outcomes was also explored. No effects were observed of GABA on parameters of glycemic control (HbA1c, insulin, glucagon, mean amplitude of glycemic excursions, and standard deviation (measured with flash glucose monitoring)) or cardiovascular health (blood pressure, 24-hour blood pressure, circulating triglycerides, cholesterol). There was also no acute effect (immediately after intake) of GABA on the postprandial glucose response or blood pressure. A decrease in fasting plasma glucose and self-reported sleep quality as compared to placebo was observed although this was no longer significant after a correction for the false discovery rate.

In this general discussion, the potential of GABA food sources will be evaluated. First, paying specific attention to the role of tomato and potato as GABA sources. Next, it will be evaluated to what extent the existing evidence substantiates any health effects of GABA. Subsequently, the clinical relevance of the findings and weaknesses in the used research methods will be discussed. Finally, recommendations for further research will be provided to arrive at a conclusion about the potential of GABA as a dietary plant component with health promoting properties.

GABA as a dietary component from food sources

Since GABA is best known as an inhibitory neurotransmitter, research on GABA as a component of the diet has so far received less attention. Diet is a natural source of GABA and this thesis shows that specifically potato and tomato can contribute to GABA intake (chapter 2). The GABA and glutamic acid content of different tomato and potato genotypes had not previously been screened so extensively. In this thesis, these findings were also translated to humans by determining the relative bioavailability of GABA from a food matrix. GABA from food sources is absorbed from the gut with similar

efficiency as from a GABA supplement. GABA from food sources is therefore likely to have similar physiological effects as when taken as a single compound (chapter 4).

In a screening of various tomato and potato genotypes, a 14-fold and 11-fold difference in GABA content between different genotypes were observed, respectively (chapter 2). Differences in concentration were stable across years, indicating that genetic variation has a relatively large effect on GABA content. Lycopene (an important carotenoid in tomatoes) for example, only has a 4.75-fold variation between genotypes [1]. GABA has not been a breeding target before, and it is therefore likely that the GABA content could be increased significantly more in novel genotypes, obtained by combining different alleles that promote GABA content.

In plants and animals, GABA is metabolized via the GABA shunt which bypasses two steps in the tricarboxylic acid (TCA) cycle [2]. GABA is made by decarboxylation of glutamic acid, catalyzed by glutamic acid decarboxylase (GAD), and can subsequently be deaminated by GABA transaminase (GABA-T) into succinic semialdehyde. From succinic semialdehyde, succinate is formed by the enzyme succinic semialdehyde dehydrogenase (SSADH) and funneled into the TCA cycle. The large natural variation in GABA content is most directly modulated by differences in glutamic acid decarboxylase (GAD) or GABA transaminase (GABA-T) activity, but steps further upstream and downstream will clearly also be relevant.

In the mature green tomato, about 50% of the total free amino acids is GABA. This accumulation of GABA during fruit development is accompanied by an increase in GAD activity [3]. The increase in GABA content during development is important for maintaining the intracellular pH and the protection of immature seeds against pests [4]. After the mature green stage, the GABA content and GAD activity decrease in tomatoes. During the ripening, GABA is used as an energy source and the increase in glutamic acid gives an "umami" taste that helps in the dispersion of seeds [5]. The changes in GABA content during the ripening process were confirmed in the tomato genotypes that were investigated in this thesis (chapter 2). Although GABA content in potatoes has been less well investigated, GABA is also increased in potato tubers after exposure to stressors like those occurring during storage [6]. All in all, the accumulation of GABA seems to serve a physiologically relevant purpose in plants.

The screening revealed a potato (cv. 'Riviera', 759 µg/g FW) and a tomato (cv. 'Madara', 994 µg/g FW) cultivar with an exceptionally high GABA content (chapter 2). In Madara, the GABA content decreased less during ripening, as compared to some other genotypes. Possibly, differences in GAD or GABA-T activity or enzyme characteristics caused the higher accumulation of GABA in the Riviera and Madara cultivars. Another possibility is a difference in synthesis and metabolism further upstream and downstream in the GABA shunt. A high GABA content is for example also observed in tomatoes carrying a gene-edited GAD gene. In these tomatoes, the GABA accumulates up to 1250 µg/g FW due to the deletion of the product inhibition domain [7]. The identified genotypes in chapter 2, therefore, seem to already have some

characteristics that lead to the accumulation of GABA, and the GABA content might be increased even further with targeted breeding.

Translating GABA content in food sources to human consumption

The content levels described above may seem somewhat abstract, but when put into the context of the average daily tomato and potato consumption, it becomes clear that selected tomato and potato cultivars could make a relevant contribution to the daily GABA intake. Based on the average potato and tomato consumption in the European Union, the daily GABA intake would normally be ca. 97 mg (75 mg from potato and 22 mg from tomato based on the genotype averages from chapter 2) [8,9]. If instead exclusively Riviera potatoes and Madara tomatoes would be consumed, this would amount to ca. 263 mg (173 mg from potato and 90 mg from tomato), which approaches levels that are usually reached with GABA supplements only.

Some other food products are also considered potentially interesting GABA sources. However, these are usually not consumed in similarly large quantities as tomatoes and potatoes. Melons for example, can contain up to 3000 µg/g FW GABA, but the average Dutch individual only consumes 2.3 grams of melon per day [10,11]. This means that high GABA-containing melons will contribute only 6.9 mg of GABA to the daily GABA intake. Dishes like salami pizza or spaghetti with tomato sauce that are found in the supermarket contain 98 and 59 mg GABA, respectively [12]. Depending on the products used, it can be roughly estimated that the usual GABA intake per day can vary anywhere between 50 to 200 mg (estimated based on food diaries). An additional, ~170 mg GABA a day from switching to high GABA tomato and potato varieties would therefore be a substantial increase.

However, the GABA content that is measured in unprepared foods does not directly translate to the amount of GABA that is consumed. Tomatoes often, and potatoes always, undergo various preparation methods before consumption. GABA losses occur during domestic preparation processes, but not more than 20% (chapter 2). Therefore, GABA is relatively stable during domestic cooking, which is in contrast to other components including vitamin C, of which the content in potatoes can be reduced by 20-80% during different cooking methods [13]. Interestingly, we observed that the GABA content even tended to increase while cooking whole tomatoes (chapter 2). Further research into possibilities to increase GABA content with specific treatments might be worthwhile. GABA is known to accumulate in response to several stressors like cold, heat, and salt [14]. Heat-drying up to a temperature of 40 degrees Celsius can increase GABA content for example, possibly through a different ratio of GAD/GABA-T activity [15]. Overall, potatoes and tomatoes can be considered sources of GABA, even after preparation.

Overall, while GABA content is already relatively high in tomatoes and potatoes, there seem to be various possibilities to increase the GABA content in tomatoes and potatoes. The large variation between genotypes and the reproducibility of this variation between

years, suggests a strong potential for targeted breeding with multiple genes to potentially generate higher levels. Next to this, there is potential for pre- and post-harvest enrichment strategies like applying environmental stress (temperature, drought, salt). Which genetics are at the basis of the high GABA tomato and potato genotypes that were described, should be further investigated. The findings in this thesis suggest that novel genetic loci are at the basis of the high GABA Madara tomato. Here, a stronger increase in glutamic acid during ripening was observed, in conjunction with a slower decrease in GABA content. By comparison, in the DG03-9 variant that was previously investigated, the GABA content decreased less, while the glutamic acid content developed similarly to other tomatoes with a lower GABA content [5]. Glutamic acid is the precursor for GABA, and this abundance of glutamic acid in the Madara tomato could be related to the relatively high GABA content. Combining different genes carrying relevant mutations that boost GABA content could be a good strategy. Chapter 2 shows that tomatoes and potatoes could be good sources of dietary GABA and might become even better sources in the future. Notwithstanding this, the relevance of these findings is obviously dependent on whether GABA has beneficial health effects.

From GABA consumption to bioavailability for humans

The first step in translating the GABA content of food products to potential health effects in humans is to determine the oral bioavailability of GABA. It might be that the GABA that is consumed via the diet is not directly translated to an elevated GABA concentration in the body. While local biological activity in the GI tract might be possible, data from previous studies suggest that if GABA has an effect on health, this will be associated with effects in tissues including the endocrine pancreas. Previously, it was shown that intake of a GABA supplement led to rapid absorption into the blood [16]. In this experiment, a relatively high dose of 2 grams of GABA was given. In chapter 4 it is shown that GABA is also rapidly absorbed from a liter of pureed tomato containing 888 mg of GABA [17]. In the same study, the plasma kinetics of GABA from pureed tomato were compared to the plasma kinetics after administering GABA dissolved in a liter of water. On average, the plasma kinetics were similar. This demonstrates that when GABA is consumed as part of a (tomato) food matrix, it is equally effectively absorbed from the gut into the systemic blood circulation and could potentially elicit the same health effects as GABA supplements.

However, it remains unclear what portion of an orally administered amount of GABA reaches the systemic circulation and even more elusive is the amount of GABA that reaches the target tissues. Kinetic parameters have not been collected after intravenous administration to humans. Only in horses has the area under the curve after intravenous administration been compared to that after oral administration [18]. There, the fraction of GABA that circulated in blood after oral administration, as compared to intravenous administration, was 9.8%. This suggests that GABA is not fully absorbed and/or rapidly metabolized, distributed, and/or excreted. Studies with

labeled GABA in which GABA and its metabolites are measured in plasma, urine, and feces, would be useful to predict bioavailability in humans [19].

Unlike its precursor glutamic acid, which is almost completely metabolized in enterocytes, in vitro experiments suggest that GABA is readily absorbed across the intestinal barrier [20–22]. The proton-dependent transport across the apical membrane is mediated via PAT1, which accepts mostly small non-polar amino acids [23]. Studies in which GABA is intravenously injected into mice suggest that GABA is very rapidly metabolized and excreted. After injection, the abundance of GABA in the blood was relatively low compared to other tissues, and it was distributed within 3 minutes to the kidney, the liver, the pineal gland (after 6 minutes) and the pituitary gland [24]. In the liver, where GABA transaminase activity is high, this enzyme converts GABA into succinate [25]. Taken together, the evidence suggests that the presumed low bioavailability of GABA is mainly occurring via rapid metabolism and excretion, while it may also be distributed to tissues where it could potentially have physiologically relevant effects.

In a 1986 study by Nurnberger et al. [26], GABA was injected into human volunteers. Even with the lowest dose of ~7.5 mg adverse effects like flushing, anxiety, and uncomfortable feelings were observed. Therefore, a low bioavailability is consistent with the lack of such adverse effects seen after oral administration, like the consumption of GABA-containing foods. In line with this, the fasting GABA concentration in plasma was shown to be fairly stable in individuals as shown in chapter 4. Over 4 consecutive weeks, the within-person variation in GABA plasma concentration was 5.8 CV% on average, while between individuals the GABA plasma concentration ranged from 14.2 to 20.3 ng/mL (chapter 4). In addition, the fasting GABA plasma concentration does not seem to reflect the dietary GABA intake. After 3 months of GABA supplementation, no increase in fasting plasma GABA concentration was observed (chapter 5). Remarkably, studies have suggested that the fasting GABA plasma concentration might be associated with the presence of mental disorders. A lower GABA plasma concentration (~10 ng/mL or lower) was found to be related to depression and bipolar disorder [27,28]. All in all, GABA plasma levels seem to be very well controlled and not easily changed via oral GABA administration. Plasma levels are therefore not a good biomarker of dietary GABA intake but might reflect concentrations in the central nervous system.

The latter would be supported by the fact that differences in brain GABA levels are shown to be related to differences in plasma GABA levels [29]. Although GABA seems to be unable to cross the blood-brain barrier from blood to the brain, GABA is actively transported from the cerebrospinal fluid to the blood circulation [30,31]. Another hypothesis is that circulating GABA levels can be influenced by the gut microbiota that consume and/or produce GABA [32]. In humans, it was shown that a fecal transplant from lean donors to obese individuals, increased GABA plasma levels to a larger extent than all other measured metabolites [33]. In germ-free mice, the GABA concentration

in blood and feces was far lower than in mice in which the microbiota were re-introduced, while cerebral GABA concentrations were similar [34]. Later, the same authors showed that GABA was increased in feces and cardiac blood after inoculation with feces, but not in the colonic tissue or portal vein, suggesting that GABA produced by the microbiota is not readily transported from the colon to the systemic circulation [35]. Therefore, the increase in GABA levels in cardiac blood after inoculation with microbiota is unexplained and most likely not mediated via the production of GABA by the microbiota. Other pathways should be considered, like an indirect effect of the microbiota on GABA levels in the systemic circulation via the gut-brain axis or the production of other metabolites [36]. Potentially, this could lead to other ways to increase GABA plasma levels. In chapter 4 it was already shown that consuming glutamic acid (precursor of GABA) does not increase the circulating GABA levels. However, further research should clarify whether and how specific microbiota can increase GABA levels in plasma. Next to glutamic acid, there might be compounds or food products that can modulate the microbiota composition to increase GABA levels.

From the results described in chapter 4, it becomes clear that GABA is rapidly taken up in the blood from a solution in water and from a tomato food matrix. However, after a transient elevation, circulating GABA concentrations show a sharp drop and seem to be strictly controlled (chapters 4 and 5). The circulating GABA plasma concentration can therefore not be used as an exposure biomarker for habitual dietary GABA intake. Biomarkers of intake are essential for further research into the health effects of a GABA-rich diet. To identify possible biomarkers of GABA intake, it should be determined which conjugates or metabolites of GABA are elevated in blood and urine directly after GABA intake and after a long-term intervention. While within an individual, the plasma GABA concentration is strictly controlled, the plasma kinetics differed substantially between individuals. For further research, it would be interesting to dive into these interindividual differences. A deeper understanding of the causes of these interindividual differences would provide the opportunity to personalize future advice on GABA intake. Research could focus on the effects of genetic variants that are involved with GABA uptake, like SNP's in intestinal and hepatic transporters of GABA. The interindividual differences should also be investigated in relation to sex, age and health status, to be able to translate the results to a wider population. In addition, studies with labeled GABA would be helpful to better establish the distribution, metabolism, and excretion of GABA in humans. In animals, studies with labeled GABA would also be of added benefit to investigate whether oral GABA crosses the blood-brain barrier, which is still a controversial topic. Together with the results presented in this thesis, this would be helpful in gaining a deeper understanding of how, at which sites and at which dose oral GABA can have relevant physiological effects

GABA supplementation does not lead to an improvement in overall insulin resistance and glucose tolerance in individuals with prediabetes

So although tomato and potato seem to be relevant food sources of GABA, and plasma GABA concentrations increase after the intake of pureed tomato (chapter 4), it is still unclear whether these transiently elevated GABA plasma levels after food intake are associated with health benefits. As is shown in chapter 5, GABA does not accumulate in plasma after a prolonged period of increased oral intake. However, literature (mostly rodent studies) does indicate systemic effects of oral GABA intake in the form of supplements. In this thesis, results are presented of the first longer-term study that attempted to translate these findings to a human situation. We explored the effects of 3 months GABA supplementation on markers of glycemic control, cardiovascular health, and sleep quality in individuals with prediabetes. We observed no post-intervention effect of GABA supplementation on the postprandial glucose, insulin, and glucagon response. We observed no effects of GABA on any other of the parameters that we assessed. This included an assessment of the acute effects of GABA on the glucose response and blood pressure, directly after intake. We did show some indications that GABA supplementation might decrease the fasting blood glucose and improve self-reported sleep quality with a greater effect in individuals with poor sleep quality.

In one of the first studies in which GABA was administered orally to humans, Cavagnini et al. (1982) showed that a single dose of 5 or 10 grams of GABA increased the circulating insulin and glucagon levels, but did not change the blood glucose levels [37]. A later study by Li et al. (2015) confirmed these findings. In this study, GABA was administered both as a single dose of 2 grams and as a repeated dose of 2 grams, 3 times a day, for a week [16]. The insulin concentration was increased under both fasting and post-meal conditions. Glucagon was only raised under fasting conditions, 4 hours after GABA intake. However, no effect on glucose levels was observed in these healthy individuals. These studies only looked at changes in glucose, insulin, and glucagon response directly after GABA intake. In line with this, we show that, even in individuals with impaired glucose tolerance, GABA does not influence the postprandial glucose response directly after a single dose of 0.5 grams GABA (chapter 5). However, the acute effects of GABA on glucagon and insulin levels were not investigated in this thesis. The primary interest of the study was the post-intervention effect of 3 months of GABA supplementation. On the day of the post-intervention measurements, GABA was not administered. Thus, the results show that regular exposure to oral doses of GABA does reduce the fasting blood glucose, but does not influence the post-intervention postprandial glucose, insulin, and glucagon response when GABA is no longer administered.

No human intervention studies are available that investigated the post-intervention effects of GABA. The effects of more long-term GABA supplementation have only been investigated in rodents. Most of these studies used models for either type 1 or type 2

diabetes. These studies show beneficial effects of GABA (orally or intravenously administered) on glucose tolerance and insulin sensitivity [38–43]. Most useful for comparison with the results described in chapter 5 are the rodent studies that investigated the effects of oral GABA supplementation in prediabetic and high-fat diet models. Tian et al. (2011) and Fu et al. (2022) assessed the effects of GABA supplementation in a type 2 diabetic, high-fat diet induced mouse model [44,45]. In the study by Tian et al. (2011) the mice were fed a high-fat (60%) diet for 20 weeks and were given either plain water or GABA (2 mg/mL) added to the drinking water [44]. In that study, the effects of GABA during the progression to diabetes were investigated and a reduction in body weight, lower fasting blood glucose, improved glucose tolerance, and insulin sensitivity were observed after 10 weeks. In a second experiment, the same authors studied the effects of 12 weeks of GABA supplementation after the onset of diabetes and again showed a reduction in body weight and improvements in fasting blood glucose, glucose tolerance, and insulin sensitivity as compared to the placebo. Fu et al. (2022) did a similar study in which diabetes was induced with a high-fat (60%) diet for 14 weeks, during these 14 weeks GABA (6 mg/mL) was administered in the drinking water [45]. The mice remained weight stable but were more glucose tolerant and insulin sensitive after GABA administration. These two studies show that long-term GABA supplementation does seem to affect glucose tolerance and insulin sensitivity in type 2 diabetic rodents.

At the same time, Untereiner et al. (2018) did not observe such effects in a prediabetic mouse model [46]. Although the mice in their experiments were fed a high-fat diet for 10 weeks, they were not diabetic. During these 10 weeks, GABA (6 mg/mL) was administered via drinking water. The investigators observed no difference in glucose tolerance and insulin sensitivity as compared to the control. However, in healthy mice (supplemented with GABA for 10 weeks) on a chow diet, they did observe an improvement in glucose tolerance and an increase in insulin secretion during an OGTT. In addition, an increase in pancreatic β-cell proliferation was observed in these healthy mice, which might explain the increased insulin secretion. Such a lack of effect of GABA supplementation on glucose tolerance and increased insulin in prediabetic mice, while showing an effect in healthy mice as described by Untereiner et al. (2018), would at least be partly in line with our findings. As shown in chapter 5, no improvement in glucose tolerance by GABA was observed in individuals with prediabetes. The study by Untereiner et al. (2018), focused on an increase in β-cell proliferation as the primary mechanism of action to explain the effects of GABA in healthy rodents [46]. However, one may speculate that in individuals with prediabetes, additional stimulation of β-cell proliferation might not be possible. In the prediabetic stage before the progression to diabetes, the tissues first become insulin resistant. This initially already leads to an increase in pancreatic β-cell mass to increase insulin secretion [47]. At first, this compensates for insulin resistance and keeps the glucose tolerance below the diabetic range. Eventually, this leads to β-cell failure, a reduction in insulin production, and a progression to diabetes [48]. The already high state of β-cell proliferation might explain why Untereiner et al. (2018) observed no beneficial effects of GABA in prediabetic mice

and why no beneficial effects on glucose tolerance and insulin resistance were observed in humans with prediabetes in chapter 5.

In the studies by Fu et al. (2022) and Tian et al. (2011), the authors investigated inflammation as a possible mechanism of action. After GABA supplementation in diabetic mice, fewer macrophages were infiltrated in the adipose tissue [44,45]. If GABA could reduce the infiltration of macrophages in individuals with prediabetes, this would potentially be useful in reducing insulin resistance. Inflammation is hypothesized to play a large role in insulin resistance. Metabolic stress caused by overnutrition leads to insulin resistance in the tissues. This stress both induces and is increased by inflammation [49]. In adipose tissue, for example, adipocytes expand due to overnutrition. This leads to hypoxia and cell death, which recruits macrophages to the adipose tissue and fuels an inflammatory response. GABA has been shown to dampen the inflammatory response of immune cells by binding to their $GABA_A$ receptors [50]. However, the effect of GABA on these immune cells has only partly been established and varies based on immune cell type and expression of receptor subtypes [51]. In chapter 5, changes in inflammation in tissues like the adipose tissue were not assessed. However, no reduction in insulin resistance was observed after 3 months of GABA supplementation (chapter 5), which we would have expected as a consequence of a reduction in inflammation. From the scarce evidence that is available, it can be hypothesized that GABA may influence β-cell proliferation and thereby insulin and glucagon secretion in healthy and type 2 diabetic rodents/humans. However, in individuals with prediabetes, no effect of GABA on insulin sensitivity and glucose tolerance is observed, since the already proliferating β-cells do not benefit from the additional stimulus that GABA might provide. In addition, the effects of GABA on inflammation should be further examined in humans, for example by examining fat tissue biopsies.

Effects of GABA supplementation on fasting blood glucose and sleep quality

Although in chapter 5 no changes were observed in glucose tolerance, insulin sensitivity nor any of the other parameters of glycemic control, the reduction seen in fasting blood glucose, of 0.22 mmol/L (95% CI: 0.397, 0.045) does seem to indicate an effect on glucose homeostasis. It should be noted that this was no longer a significant finding after correction for the false discovery rate. The observed reduction in fasting glucose level was independent of the individual's fasting blood glucose level before the intervention. This effect size is of the same order of magnitude as found in other studies investigating bioactive food components or lifestyle interventions [52–54]. For example, a meta-analysis showed that green tea catechins significantly decrease fasting blood glucose by 0.082 mmol/L on average [54]. In addition, Schutte et al. (2022) recently reported that a 12-week, 25% energy restriction trial that successfully induced weight loss, led to a reduction in fasting glucose concentration of 0.3 mmol/L in a comparable population [55]. The fasting glucose concentration is normally tightly controlled in a range from 3.9 to 6.9 mmol/L. Even small changes can

have substantial effects on health outcomes. For prediabetes, estimates suggest that a 0.5 mmol/L increase in fasting glucose concentration would be associated with a 2- to 3-fold increased risk of progression to diabetes [56–58]. In this regard, the results of the present intervention could be considered clinically relevant. However, unlike lifestyle interventions, GABA supplementation only decreases fasting blood glucose and none of the other parameters related to glycemic control.

Although lifestyle interventions would be preferable and further research is necessary to verify this, it is still interesting to speculate about potential mechanisms of action that could explain this reduction in fasting blood glucose. Our observation suggests that GABA is somehow involved in the regulation of endogenous glucose production during fasting. During the night, in absence of dietary intake, glucose homeostasis is regulated by endogenous glucose production [59]. Endogenous glucose production consists of glycogenolysis (release of glucose from the stored glycogen) and gluconeogenesis (new glucose production). In healthy individuals, glycogenolysis slowly decreases during the night and gluconeogenesis remains stable [60]. In individuals with type 2 diabetes, endogenous glucose production is higher, leading to elevated fasting glucose. During the night, glycogenolysis decreases to a level similar to that in healthy controls, while gluconeogenesis increases to a level higher than in healthy controls.

To understand how GABA could potentially modulate the regulation of endogenous glucose production, it is useful to consider how it is usually regulated. Glycogenolysis takes place in the liver, muscle, and brain. It is stimulated in response to glucagon and catecholamines and inhibited by glucose. On the other hand, glycogen production is stimulated by insulin while glucocorticoids inhibit the uptake of glucose [61]. Gluconeogenesis takes place in the liver, kidneys, and intestine. Under fasting conditions, 75 – 80% of the glucose in circulation is synthesized in the liver. The remaining part is mainly produced in the kidneys [62,63]. Gluconeogenesis is also regulated by insulin, glucagon, glucocorticoids, and catecholamines [59]. Next to that, lipolysis in the adipose tissue plays a role in regulating gluconeogenesis, since glycerol is a substrate for gluconeogenesis [64]. Increased availability of this substrate through lipolysis stimulates gluconeogenesis. Insulin normally inhibits lipolysis and therefore indirectly inhibits hepatic gluconeogenesis [65]. Gluconeogenesis is also regulated by the nervous system via direct sympathetic and parasympathetic stimulation and the activation of the hypothalamus-pituitary-adrenal (HPA) axis [66,67].

Twelve weeks of GABA supplementation does not influence fasting insulin and glucagon levels (chapter 5). Therefore, there does not seem to be a sustained effect of GABA on endogenous glucose production by changes in circulating insulin and glucagon levels. One can only speculate about any other potential mechanism of action. Higher fasting glucose levels can be caused by an increase in lipolysis due to insulin-insensitive adipose tissue [59]. This increased lipolysis leads to an increased amount of circulating glycerol, the main substrate for the production of glucose in the liver. GABA

supplementation likely does not solve this underlying issue, since it decreased fasting glucose levels equally in both individuals with a normal fasting blood glucose level and an impaired fasting blood glucose level (chapter 5). In addition, GABA supplementation does not reduce circulating free fatty acid levels (data not shown), increased lipolysis in insulin resistant individuals would also lead to higher levels of circulating free fatty acids [68]. GABA could for example have an effect on the neuronal control of endogenous glucose production or have a direct effect in the organs that control endogenous glucose production. This could be either directly in the liver or in endocrine glands. Here, the focus will be on the available literature about the role of GABA in the liver, pituitary, adrenal and pineal glands.

Since gluconeogenesis mainly takes place in the liver, it is likely that the reduction in fasting blood glucose that was observed in chapter 5 is mediated via a role of GABA in the liver. Additionally, GABA is metabolized by the liver and distributed rapidly to the liver [24,25]. Hepatocytes also express GABA receptors and GABA is produced and secreted by hepatocytes [69]. Interestingly, hepatocytes express the GABA transporter GAT-2, which main function is also to transport β-alanine into the hepatocytes as gluconeogenic substrate. GABA can compete for this transporter and inhibit the uptake of β-alanine, which could potentially influence the gluconeogenic flux [70]. GABA in the liver has mainly been investigated in relation to hepatocellular carcinoma. Normally, $GABA_A$ receptor activation leads to a hyperpolarization of the hepatocyte plasma membrane [71]. Decreased GABAergic activity leads to depolarization of the membrane in tumor cells, which is linked to increased proliferation. Geisler et al. (2021) investigated the endogenous function of GABA in the liver, in relation to obesity [69]. The lipid accumulation in obesity also leads to a depolarization of the hepatocyte plasma membrane and in a depolarized state, the hepatocytes release more GABA. In their paper, Geisler et al. propose that GABA could function as a hepatokine, signaling to the central nervous system via the vagal nerve [72]. While the role of GABA in the liver is becoming more clear, it has not been investigated what the effects of exogenous GABA administration are on the gluconeogenic flux in hepatocytes directly or under the neuronal control of glucose homeostasis.

The HPA axis would also be of special interest as potential site of action for GABA, since GABA levels were shown to distribute rapidly to the pituitary gland after intravenous injection in rodents [24]. Additionally, the endocrine cells of the adrenal and pituitary glands express both functional $GABA_A$ and $GABA_B$ receptors [73]. It was shown that GABA can alter the membrane potential of these endocrine cells and thereby modulate their secretion of hormones [74,75]. Whether GABA inhibits or stimulates hormone release from these glands is still controversial [76]. Like in the pancreas, where GABA can either increase or decrease insulin secretion, GABA modulation of catecholamine release can also go either way [77]. A possibility is that the function of GABA depends on the situation and dampens the catecholamine release to prevent excessive release [78]. Whether oral GABA administration can also have a sustained influence on catecholamine release remains to be investigated. The effect of oral GABA

supplementation on growth hormone (secreted from the pituitary gland) secretion has been investigated. Cavagnini et al. (1980) administered an acute dose of 5 grams of GABA to healthy volunteers and a dose of 18 grams for a prolonged 4-day period [79]. The investigators observed an increase in growth hormone levels after the acute dose and a blunted growth hormone response after an insulin challenge.

Next to the pituitary gland, GABA was also shown to rapidly distribute to the pineal gland [24]. The pineal gland also expresses functional $GABA_A$ and $GABA_B$ receptors [80]. The pineal gland is responsible for the secretion of melatonin in response to signals from the circadian clock in the hypothalamus [81]. Melatonin levels rise a few hours before sleep, decrease a few hours after waking and stay low during the day. Recently, there is increasing interest in melatonin in relation to glucose homeostasis. In GWAS studies a SNP in the melatonin receptor (leading to increased expression of the receptor) was linked with increased fasting plasma glucose and type 2 diabetes risk [82]. However, it is still controversial whether melatonin is beneficial or deleterious in type 2 diabetes. In a conceptual framework, Garaulet et al. (2020) propose that it depends on the timeframe [83]. A concurrence of eating and high melatonin levels would be deleterious, while high melatonin levels during the night would be beneficial for glucose homeostasis. Although there seems to be a role of GABA in the pineal gland, its effect on melatonin secretion remains to be investigated [84].

Interestingly, we also observed an improvement in sleep quality after the administration of GABA as compared to a placebo (chapter 5). When interpreting this finding it should be taken into account that it was no longer significant after a correction for the false discovery rate. The literature does describe a clear association between sleep quality and glucose homeostasis [85]. Although the mechanism of action is not fully clear, the hypothalamic-pituitary-adrenal axis and sympathetic activation seem to play a role [86]. Previously, the effects of GABA on sleep quality in humans have been described in two other studies. Byun et al. (2018) investigated the effects of 300 mg GABA daily in 40 individuals with poor sleep quality (30 received GABA, 10 received a placebo) for 4 weeks [87]. They found a significant reduction in sleep latency (the time until falling asleep) in the GABA group as compared to the placebo group. Yamatsu et al. (2015) administered 100 mg GABA daily for 1 week to 8 individuals with poor sleep quality (8 received GABA and placebo in a crossover design) [88]. They observed no significant differences between the groups.

When considering receptor expression and presumed distribution of GABA, oral GABA supplementation has the potential to affect glucose homeostasis via many different mechanisms in different organs. However, evidence for effects of exogenous GABA supplementation in these systems is still lacking. As of yet, research has mainly focused on the effects of endogenous GABA, while we show that GABA is taken up in the blood after dietary consumption (chapter 4). Elucidating the mechanism of action of GABA could lead to more targeted interventions. In humans, it would be interesting to test the hypothesis that GABA supplementation somehow inhibits gluconeogenesis

in the liver or influences the circadian control of glucose production via the HPA axis or pineal gland. Different timing of GABA supplementation (during the day and during the night) in combination with drawing blood at multiple time points during the day and night would give more insight into its mechanism. Levels of melatonin, catecholamines, and glucocorticoids could be determined during the day and night.

Recommendations for further research

The goal of this thesis was to explore whether GABA has any potential as a dietary food component with health benefits. Since the effects of nutrition are often too subtle to cure disease, we focused on prevention as the main goal. At the same time, preventative capacity is difficult to investigate since chronic disease develops over time and the subtle effects of nutrition require long-term compliance to have a substantial effect on prevention. A randomised, placebo-controlled clinical trial is still the best way to investigate the health effects of dietary components. However, due to time and financial constraints, a surrogate measure for disease risk has to be used to determine effects over a shorter time frame. The oral glucose tolerance test and blood pressure are examples of good surrogate measures that predict future diabetes and cardiovascular disease development. In chapter 5, we assume that the effects of GABA on these surrogate markers predict a future effect on disease development in these at-risk individuals. However, we did not measure the effects of GABA for a long enough period of time to determine whether actual disease development is affected.

We show that GABA supplementation reduces fasting blood glucose and improves sleep quality, but has no effect on any of the other parameters that were measured. However, the observed effects cannot immediately be translated to all populations and settings, this requires additional research. Since rodent studies point to effects on β-cell function, the effects of GABA should also be determined in individuals with type 2 diabetes. We hypothesize that the effects of GABA would be more pronounced in these diseased individuals. In addition, in analyzing interactions between outcome values and their respective pre-intervention values, it was observed that the effect of GABA on blood pressure and sleep quality was dependent on the pre-intervention state of the individuals. The effect of GABA was larger in individuals with poor sleep quality and hypertension. Although for blood pressure this was no longer significant after correction for multiple testing, to follow up, the study can be repeated in individuals with hypertension, with blood pressure as a primary outcome. Sleep quality was also described as an exploratory secondary outcome, it would therefore be of additional interest to set up a study to determine the effects of GABA on sleep quality in poor sleepers. This should include more objective measures of sleep quality. The effects of GABA on sleep quality and hypertension are of particular interest since GABA-enriched food products are already on the market in for example Japan, claim benefits in these areas.

Next to blood pressure and sleep quality, multiple exploratory secondary outcomes were assessed. Method triangulation with standardized research methods in

combination with real-world data was applied to increase the validity of the research findings. However, the real-world data collected with ambulatory blood pressure monitors and flash glucose monitoring suffered from large interindividual differences. To use this data beyond exploring secondary outcomes, a larger sample size is required. Flash glucose monitoring is a promising development for clinical practice but remains less precise than glucose levels measured in blood. It is an easy way to explore the effects of an intervention in a real-life setting, by gathering a large amount of data from a large number of people at a relatively low effort for the participants. However, variation in the behavior of participants does not allow for capturing small differences in a clinical trial.

As is shown in chapter 2, GABA is present in relevant food products and by choosing specific food products/varieties the daily GABA intake can be substantially increased. In addition, GABA from a food matrix can also be taken up into the bloodstream (chapter 4). However, the data from our studies do not support the hypothesis that increased GABA consumption via food can lead to substantial health benefits. In chapter 5 we investigated the effect of supplementing three times daily 500 mg of GABA for 3 months. Although further research is necessary to determine the optimal effective dose of GABA, increasing this dose might have resulted in more pronounced health effects. However, the 1500 mg that was administered per day is already substantially higher than the ~170 mg that the dietary GABA intake could be increased with, by choosing high GABA-containing potatoes and tomatoes. Although the daily GABA intake could most likely become higher by further adjusting diet choices and plant breeding to maximize the GABA content, it does not seem realistic to target an additional 1500 mg a day. Most likely, the health effects of increasing daily GABA content through food will therefore be smaller. Currently, it would be difficult to investigate the effects of a GABA-enriched diet, since data on the GABA content of many food products is not available. For further research, it would therefore be necessary to determine the GABA content of more food products. Currently, it is still unknown how much GABA people usually consume and what the variation is between individuals. As for most bioactive food components, GABA is not added to the food composition tables and is not taken along in routine analysis of foods. As we show in chapter 2, it would also be necessary to take into account the effect of genotype on food composition and cooking methods when analyzing the GABA content of food products.

Conclusion

While this thesis uncovered some of the potential of GABA as a bioactive food component, currently the evidence is not sufficient to recommend an increased dietary intake of GABA. The research does indicate that tomato and potato genotypes are potentially good sources of GABA. Especially since the consumption of potatoes and tomatoes is high around the world and GABA content is only moderately reduced during the processing of raw fruits and tubers. In addition, GABA is taken up from a tomato

food matrix into the blood, equally well as from a solution in water. Finally, we give some indications for beneficial health effects of GABA. Although modest when compared to the efficacy of lifestyle interventions in improving overall health. This thesis also leaves many questions for further research into the health effects of GABA. Further investigations should focus first on elucidating the mechanisms of action that explain the effects of exogenously administered GABA. Gaining an understanding of how, where and in what way GABA can influence tissue function, can drive further, more focused, research that investigates the health effects of GABA and a GABA-rich diet.

References

[1] D.R. Panthee, P. Perkins-Veazie, D. Randall, A.F. Brown, Lycopene Estimation in Tomato Lines Using Infrared Absorbance and Tomato Analyzer, Http://Dx.Doi.Org/10.1080/19315260.2012.715324. 19 (2013) 240–255. https://doi.org/10.1080/19315260.2012.715324.

[2] N. Bouché, H. Fromm, GABA in plants: Just a metabolite?, Trends Plant Sci. 9 (2004) 110–115. https://doi.org/10.1016/j.tplants.2004.01.006.

[3] D. Rolin, P. Baldet, D. Just, C. Chevalier, M. Biran, P. Raymond, NMR study of low subcellular pH during the development of cherry tomato fruit, Functional Plant Biology. 27 (2000) 61–69. https://doi.org/10.1071/PP99051.

[4] H.S. Seifi, K. Curvers, D. De Vleesschauwer, I. Delaere, A. Aziz, M. Höfte, Concurrent overactivation of the cytosolic glutamine synthetase and the GABA shunt in the ABA-deficient sitiens mutant of tomato leads to resistance against Botrytis cinerea, New Phytologist. 199 (2013) 490–504. https://doi.org/10.1111/NPH.12283.

[5] M. Takayama, H. Ezura, How and why does tomato accumulate a large amount of GABA in the fruit?, Front Plant Sci. 6 (2015) 612. https://doi.org/10.3389/fpls.2015.00612.

[6] A. Pęksa, J. Miedzianka, A. Nemś, E. Rytel, The free-amino-acid content in six potatoes cultivars through storage, Molecules. 26 (2021) 1322. https://doi.org/10.3390/molecules26051322.

[7] S. Nonaka, C. Arai, M. Takayama, C. Matsukura, H. Ezura, Efficient increase of ɣ-aminobutyric acid (GABA) content in tomato fruits by targeted mutagenesis, Scientific Reports 2017 7:1. 7 (2017) 1–14. https://doi.org/10.1038/s41598-017-06400-y.

[8] Directorate-General for Agriculture and Rural Development, EU AGRICULTURAL OUTLOOK FOR THE AGRICULTURAL MARKETS AND INCOME 2021-2031, 2021. https://doi.org/10.2762/753688.

[9] R. Wijesinha-Bettoni, B. Mouillé, The Contribution of Potatoes to Global Food Security, Nutrition and Healthy Diets, American Journal of Potato Research 2019 96:2. 96 (2019) 139–149. https://doi.org/10.1007/S12230-018-09697-1.

[10] C. van Rossum, E. Buurma-Rethans, C. Dinnissen, M. Beukers, H. Brants, M. Ocké, The diet of the Dutch : Results of the Dutch National Food Consumption Survey 2012-2016, (2020). https://doi.org/10.21945/RIVM-2020-0083.

[11] T. Toyoizumi, S. Ohba, K. suke Fujii, A. Ikegaya, H. Matsuura, T. Nakajima, Differential GABA concentration gradients are present in the edible parts of greenhouse melon (Cucumis melo L.) during all four seasonal croppings, Biosci Biotechnol Biochem. 83 (2019) 330–338. https://doi.org/10.1080/09168451.2018.1537774.

[12] M. Hermanussen, U. Gonder, C. Jakobs, D. Stegemann, G. Hoffmann, Patterns of free amino acids in German convenience food products: marked mismatch between label information and composition, European Journal of Clinical Nutrition 2010 64:1. 64 (2009) 88–98. https://doi.org/10.1038/ejcn.2009.116.

[13] S.L. Love, J.J. Pavek, Positioning the potato as a primary food source of vitamin C, American Journal of Potato Research. 85 (2008) 277–285. https://doi.org/10.1007/S12230-008-9030-6/TABLES/4.

[14] A.M. Kinnersley, F.J. Turano, Gamma Aminobutyric Acid (GABA) and Plant Responses to Stress, Https://Doi.Org/10.1080/07352680091139277. 19 (2010) 479–509. https://doi.org/10.1080/07352680091139277.

[15] Y. Takahashi, T. Sasanuma, T. Abe, Accumulation of gamma-aminobutyrate (GABA) caused by heat-drying and expression of related genes in immature vegetable soybean (edamame), Breed Sci. 63 (2013) 205–210. https://doi.org/10.1270/JSBBS.63.205.

[16] J. Li, Z. Zhang, X. Liu, Y. Wang, F. Mao, J. Mao, X. Lu, D. Jiang, Y. Wan, J.-Y. Lv, G. Cao, J. Zhang, N. Zhao, M. Atkinson, D.L. Greiner, G.J. Prud'homme, Z. Jiao, Y. Li, Q. Wang, Study of GABA in Healthy Volunteers: Pharmacokinetics and Pharmacodynamics, Front Pharmacol. 6 (2015) 260. https://doi.org/10.3389/fphar.2015.00260.

[17] T.H. de Bie, M.G.J. Balvers, R.C.H. de Vos, R.F. Witkamp, M.A. Jongsma, The influence of a tomato food matrix on the bioavailability and plasma kinetics of oral gamma-aminobutyric acid (GABA) and its precursor glutamate in healthy men, Food Funct. (2022). https://doi.org/10.1039/D2FO01358D.

[18] H.K. Knych, S.J. Steinmetz, D.S. Mckemie, S.S. J, D.S. Endogenous concentrations, H.K. Knych, K. Maddy Equine, Endogenous concentrations, pharmacokinetics, and selected pharmacodynamic effects of a single dose of exogenous GABA in horses, J Vet Pharmacol Ther. 38 (2015) 113–122. https://doi.org/10.1111/JVP.12146.

[19] H. Ohtani, R. Iwata, A. Imaoka, T. Akiyoshi, Estimation of absolute oral bioavailability without performing an intravenous clinical study, Drug Metab Pharmacokinet. 38 (2021) 100392. https://doi.org/10.1016/J.DMPK.2021.100392.

[20] D.T. Thwaites, L. Basterfield, P.M.J. McCleave, S.M. Carter, N.L. Simmons, Gamma-aminobutyric acid (GABA) transport across human intestinal epithelial (Caco-2) cell monolayers, Br J Pharmacol. 129 (2000) 457–464. https://doi.org/10.1038/sj.bjp.0703069.

[21] S.P. Haÿs, J.M. Ordonez, D.G. Burrin, A.L. Sunehag, Dietary Glutamate Is Almost Entirely Removed in Its First Pass Through the Splanchnic Bed in Premature Infants, Pediatr Res. 62 (2007) 353–356. https://doi.org/10.1203/PDR.0b013e318123f719.

[22] C.U. Nielsen, M. Carstensen, B. Brodin, Carrier-mediated γ-aminobutyric acid transport across the basolateral membrane of human intestinal Caco-2 cell

monolayers, European Journal of Pharmaceutics and Biopharmaceutics. 81 (2012) 458–462. https://doi.org/10.1016/j.ejpb.2012.03.007.

[23] M. Boll, M. Foltz, C.M.H. Anderson, C. Oechsler, G. Kottra, D.T. Thwaites, H. Daniel, Substrate recognition by the mammalian proton-dependent amino acid transporter PAT1, Mol Membr Biol. 20 (2003) 261–269. https://doi.org/10.1080/0968768031000100759.

[24] E. Kuroda, M. Watanabe, T. Tamayama, M. Shimada, Autoradiographic distribution of radioactivity from 14C-GABA in the mouse, Microsc Res Tech. 48 (2000) 116–126. https://doi.org/10.1002/(SICI)1097-0029(20000115)48:2<116::AID-JEMT7>3.0.CO;2-P.

[25] P. Ferenci, T. Stehle, J. Ebner, R. Schmid, D. Häussinger, Uptake and catabolism of γ-aminobutyric acid by the isolated perfused rat liver, Gastroenterology. 95 (1988) 402–407. https://doi.org/10.1016/0016-5085(88)90497-0.

[26] J.I. Nurnberger, W.H. Berrettini, S. Simmons-Alling, J.J. Guroff, E.S. Gershon, Intravenous GABA administration is anxiogenic in man, Psychiatry Res. 19 (1986) 113–117. https://doi.org/10.1016/0165-1781(86)90004-1.

[27] F. Petty, G.L. Kramer, M. Fulton, F.G. Moeller, A.J. Rush, Low Plasma GABA Is a Trait-Like Marker for Bipolar Illness, Neuropsychopharmacology. 9 (1993) 125–132. https://doi.org/10.1038/npp.1993.51.

[28] F. Petty, G.L. Kramer, M. Fulton, L. Davis, A.J. Rush, Stability of plasma GABA at four-year follow-up in patients with primary unipolar depression, Biol Psychiatry. 37 (1995) 806–810. https://doi.org/10.1016/0006-3223(94)00226-S.

[29] S. Uhlhaas, H. Lange, J. Wappenschmidt, K. Olek, Free and conjugated CSF and plasma GABA in Huntington's chorea, Acta Neurol Scand. 74 (1986) 261–265. https://doi.org/10.1111/J.1600-0404.1986.TB03511.X.

[30] S.R. Snodgrass, A. V. Lorenzo, TRANSPORT OF GABA FROM THE PERFUSED VENTRICULAR SYSTEM OF THE CAT1, J Neurochem. 20 (1973) 761–769. https://doi.org/10.1111/J.1471-4159.1973.TB00037.X.

[31] E. Boonstra, R. de Kleijn, L.S. Colzato, A. Alkemade, B.U. Forstmann, S. Nieuwenhuis, Neurotransmitters as food supplements: the effects of GABA on brain and behavior, Front Psychol. 6 (2015) 1520. https://doi.org/10.3389/fpsyg.2015.01520.

[32] P. Strandwitz, K.H. Kim, D. Terekhova, J.K. Liu, A. Sharma, J. Levering, D. McDonald, D. Dietrich, T.R. Ramadhar, A. Lekbua, N. Mroue, C. Liston, E.J. Stewart, M.J. Dubin, K. Zengler, R. Knight, J.A. Gilbert, J. Clardy, K. Lewis, GABA-modulating bacteria of the human gut microbiota, Nat Microbiol. 4 (2019) 396–403. https://doi.org/10.1038/s41564-018-0307-3.

[33] R.S. Kootte, E. Levin, J. Salojärvi, L.P. Smits, A. v. Hartstra, S.D. Udayappan, G. Hermes, K.E. Bouter, A.M. Koopen, J.J. Holst, F.K. Knop, E.E. Blaak, J. Zhao, H.

Smidt, A.C. Harms, T. Hankemeijer, J.J.G.H.M. Bergman, H.A. Romijn, F.G. Schaap, S.W.M. Olde Damink, M.T. Ackermans, G.M. Dallinga-Thie, E. Zoetendal, W.M. de Vos, M.J. Serlie, E.S.G. Stroes, A.K. Groen, M. Nieuwdorp, Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition, Cell Metab. 26 (2017) 611-619.e6. https://doi.org/10.1016/J.CMET.2017.09.008.

[34] M. Matsumoto, R. Kibe, T. Ooga, Y. Aiba, E. Sawaki, Y. Koga, Y. Benno, Cerebral low-molecular metabolites influenced by intestinal microbiota: A pilot study, Front Syst Neurosci. 7 (2013). https://doi.org/10.3389/fnsys.2013.00009.

[35] M. Matsumoto, T. Ooga, R. Kibe, Y. Aiba, Y. Koga, Y. Benno, Colonic Absorption of Low-Molecular-Weight Metabolites Influenced by the Intestinal Microbiome: A Pilot Study., PLoS One. 12 (2017) e0169207. https://doi.org/10.1371/journal.pone.0169207.

[36] J.A. Bravo, P. Forsythe, M. V. Chew, E. Escaravage, H.M. Savignac, T.G. Dinan, J. Bienenstock, J.F. Cryan, Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve, Proc Natl Acad Sci U S A. 108 (2011) 16050–16055. https://doi.org/10.1073/PNAS.1102999108/SUPPL_FILE/PNAS.201102999SI.PDF.

[37] F. Cavagnini, M. Pinto, A. Dubini, C. Invitti, G. Cappelletti, E.E. Polli, Effects of gamma aminobutyric acid (GABA) and muscimol on endocrine pancreatic function in man, Metabolism. 31 (1982) 73–77. https://doi.org/10.1016/0026-0495(82)90029-4.

[38] I. Purwana, J. Zheng, X. Li, M. Deurloo, D.O. Son, Z. Zhang, C. Liang, E. Shen, A. Tadkase, Z.-P. Feng, Y. Li, C. Hasilo, S. Paraskevas, R. Bortell, D.L. Greiner, M. Atkinson, G.J. Prud'homme, Q. Wang, GABA promotes human β-cell proliferation and modulates glucose homeostasis, Diabetes. 63 (2014) 4197–4205. https://doi.org/10.2337/db14-0153.

[39] S. Sohrabipour, M.R. Sharifi, A. Talebi, M. Sharifi, N. Soltani, GABA dramatically improves glucose tolerance in streptozotocin-induced diabetic rats fed with high-fat diet, Eur J Pharmacol. 826 (2018) 75–84. https://doi.org/10.1016/J.EJPHAR.2018.01.047.

[40] N. Soltani, H. Qiu, M. Aleksic, Y. Glinka, F. Zhao, R. Liu, Y. Li, N. Zhang, R. Chakrabarti, T. Ng, T. Jin, H. Zhang, W.-Y. Lu, Z.-P. Feng, G.J. Prud'homme, Q. Wang, GABA exerts protective and regenerative effects on islet beta cells and reverses diabetes., Proc Natl Acad Sci U S A. 108 (2011) 11692–7. https://doi.org/10.1073/pnas.1102715108.

[41] A.L. Feng, Y.-Y. Xiang, L. Gui, G. Kaltsidis, Q. Feng, W.-Y. Lu, Paracrine GABA and insulin regulate pancreatic alpha cell proliferation in a mouse model of type 1 diabetes, Diabetologia. 60 (2017) 1033–1042. https://doi.org/10.1007/s00125-017-4239-x.

[42] N. Rathwa, N. Parmar, S.P. Palit, R. Patel, R.S. Bhaskaran, A.V. Ramachandran, R. Begum, Calorie restriction potentiates the therapeutic potential of GABA in managing type 2 diabetes in a mouse model, Life Sci. 295 (2022) 120382. https://doi.org/10.1016/J.LFS.2022.120382.

[43] F. Yazdanimoghaddam, M. Ghasemi, · Hanif Teamparvar, N. Soltani, M. Aghaei, H. Rezazadeh, · Fouzieh Zadhoush, Long-term GABA administration improves FNDC5, TFAM, and UCP3 mRNA expressions in the skeletal muscle and serum irisin levels in chronic type 2 diabetic rats, Naunyn-Schmiedeberg's Archives of Pharmacology 2022. (2022) 1–12. https://doi.org/10.1007/S00210-022-02211-9.

[44] J. Tian, H.N. Dang, J. Yong, W.-S. Chui, M.P.G. Dizon, C.K.Y. Yaw, D.L. Kaufman, Oral Treatment with γ-Aminobutyric Acid Improves Glucose Tolerance and Insulin Sensitivity by Inhibiting Inflammation in High Fat Diet-Fed Mice, PLoS One. 6 (2011) e25338. https://doi.org/10.1371/journal.pone.0025338.

[45] J. Fu, Z. Han, Z. Wu, Y. Xia, G. Yang, Y. Yin, W. Ren, GABA regulates IL-1β production in macrophages, Cell Rep. 41 (2022) 111770. https://doi.org/10.1016/J.CELREP.2022.111770.

[46] A. Untereiner, S. Abdo, A. Bhattacharjee, H. Gohil, F. Pourasgari, N. Ibeh, M. Lai, B. Batchuluun, A. Wong, N. Khuu, Y. Liu, D. Al Rijjal, N. Winegarden, C. Virtanen, B.A. Orser, O. Cabrera, G. Varga, J. Rocheleau, F.F. Dai, M.B. Wheeler, GABA promotes b-cell proliferation, but does not overcome impaired glucose homeostasis associated with diet-induced obesity, The FASEB Journal. (2018). https://doi.org/10.1096/fj.201801397R.

[47] L.C. Alonso, T. Yokoe, P. Zhang, D.K. Scott, S.K. Kim, C.P. O'Donnell, A. Garcia-Ocaña, Glucose Infusion in Mice: A New Model to Induce β-Cell Replication, Diabetes. 56 (2007) 1792. https://doi.org/10.2337/DB06-1513.

[48] G.C. Weir, S. Bonner-Weir, Five Stages of Evolving Beta-Cell Dysfunction During Progression to Diabetes, Diabetes. 53 (2004) S16–S21. https://doi.org/10.2337/DIABETES.53.SUPPL_3.S16.

[49] M.Y. Donath, S.E. Shoelson, Type 2 diabetes as an inflammatory disease, Nature Reviews Immunology 2011 11:2. 11 (2011) 98–107. https://doi.org/10.1038/nri2925.

[50] E.L. Sparrow, S. James, K. Hussain, S.A. Beers, M.S. Cragg, Y.D. Bogdanovid, Activation of GABA(A) receptors inhibits T cell proliferation, (2021). https://doi.org/10.1371/journal.pone.0251632.

[51] A.K. Bhandage, A. Barragan, GABAergic signaling by cells of the immune system: more the rule than the exception, Cellular and Molecular Life Sciences 2021 78:15. 78 (2021) 5667–5679. https://doi.org/10.1007/S00018-021-03881-Z.

[52] Diabetes Prevention Program Research Group, Reduction in the Incidence of Type 2 Diabetes with Lifestyle Intervention or Metformin,

Https://Doi.Org/10.1056/NEJMoa012512. 346 (2002) 393–403. https://doi.org/10.1056/NEJMOA012512.

[53] X.J. Yang, S.F. Zou, Y. Xu, Y. Li, S.S. Yang, The influence of intensive lifestyle intervention on patients with isolated impaired fasting glucose: a meta-analysis, J Adv Nurs. 72 (2016) 2587–2597. https://doi.org/10.1111/JAN.13015.

[54] X.X. Zheng, Y.L. Xu, S.H. Li, R. Hui, Y.J. Wu, X.H. Huang, Effects of green tea catechins with or without caffeine on glycemic control in adults: a meta-analysis of randomized controlled trials, Am J Clin Nutr. 97 (2013) 750–762. https://doi.org/10.3945/AJCN.111.032573.

[55] S. Schutte, D. Esser, E. Siebelink, C.J.R. Michielsen, M. Daanje, J.C. Matualatupauw, H.C. Boshuizen, M. Mensink, L.A. Afman, T.W.B.F.S. team, S. Schutte, D. Esser, E. Siebelink, H. Fick, M.M. Grootte Bromhaar, Y. Wang, S.E.M. de Bruijn, M. Mars, J. Meijerink, M. Mensink, L.A. Afman, E.J.M. Feskens, M. Müller, Diverging metabolic effects of 2 energy-restricted diets differing in nutrient quality: a 12-week randomized controlled trial in subjects with abdominal obesity, Am J Clin Nutr. 116 (2022) 132–150. https://doi.org/10.1093/AJCN/NQAC025.

[56] S. Soulimane, D. Simon, J. Shaw, D. Witte, P. Zimmet, S. Vol, K. Borch-Johnsen, D. Magliano, D. Vistisen, B. Balkau, HbA1c, fasting plasma glucose and the prediction of diabetes: Inter99, AusDiab and D.E.S.I.R., Diabetes Res Clin Pract. 96 (2012) 392–399. https://doi.org/10.1016/J.DIABRES.2011.06.003.

[57] M. Tohidi, A. Baghbani-Oskouei, N.S. Ahanchi, F. Azizi, F. Hadaegh, Fasting plasma glucose is a stronger predictor of diabetes than triglyceride–glucose index, triglycerides/high-density lipoprotein cholesterol, and homeostasis model assessment of insulin resistance: Tehran Lipid and Glucose Study, Acta Diabetol. 55 (2018) 1067–1074. https://doi.org/10.1007/S00592-018-1195-Y/FIGURES/2.

[58] Y. Heianza, S. Hara, Y. Arase, K. Saito, K. Fujiwara, H. Tsuji, S. Kodama, S.D. Hsieh, Y. Mori, H. Shimano, N. Yamada, K. Kosaka, H. Sone, HbA1c 5·7–6·4% and impaired fasting plasma glucose for diagnosis of prediabetes and risk of progression to diabetes in Japan (TOPICS 3): a longitudinal cohort study, The Lancet. 378 (2011) 147–155. https://doi.org/10.1016/S0140-6736(11)60472-8.

[59] M.C. Petersen, D.F. Vatner, G.I. Shulman, Regulation of hepatic glucose metabolism in health and disease, Nature Reviews Endocrinology 2017 13:10. 13 (2017) 572–587. https://doi.org/10.1038/nrendo.2017.80.

[60] A. Basu, N. Joshi, J. Miles, R.E. Carter, R.A. Rizza, R. Basu, Paradigm Shifts in Nocturnal Glucose Control in Type 2 Diabetes, J Clin Endocrinol Metab. 103 (2018) 3801–3809. https://doi.org/10.1210/JC.2018-00873.

[61] T. Kuo, A. McQueen, T.C. Chen, J.C. Wang, Regulation of glucose homeostasis by glucocorticoids, Adv Exp Med Biol. 872 (2015) 99–126. https://doi.org/10.1007/978-1-4939-2895-8_5/FIGURES/4.

[62] M. Alsahli, J.E. Gerich, Renal glucose metabolism in normal physiological conditions and in diabetes, Diabetes Res Clin Pract. 133 (2017) 1–9. https://doi.org/10.1016/J.DIABRES.2017.07.033.

[63] M.T. Swe, A. Pongchaidecha, V. Chatsudthipong, N. Chattipakorn, A. Lungkaphin, Molecular signaling mechanisms of renal gluconeogenesis in nondiabetic and diabetic conditions, J Cell Physiol. 234 (2019) 8134–8151. https://doi.org/10.1002/JCP.27598.

[64] S.F. Previs, G.W. Cline, G.I. Shulman, A critical evaluation of mass isotopomer distribution analysis of gluconeogenesis in vivo, Am J Physiol Endocrinol Metab. 277 (1999). https://doi.org/10.1152/AJPENDO.1999.277.1.E154/ASSET/IMAGES/LARGE/AEND10717002X.JPEG.

[65] R.J. Perry, J.P.G. Camporez, R. Kursawe, P.M. Titchenell, D. Zhang, C.J. Perry, M.J. Jurczak, A. Abudukadier, M.S. Han, X.M. Zhang, H. bin Ruan, X. Yang, S. Caprio, S.M. Kaech, H.S. Sul, M.J. Birnbaum, R.J. Davis, G.W. Cline, K.F. Petersen, G.I. Shulman, Hepatic Acetyl CoA Links Adipose Tissue Inflammation to Hepatic Insulin Resistance and Type 2 Diabetes, Cell. 160 (2015) 745–758. https://doi.org/10.1016/J.CELL.2015.01.012.

[66] M.G. Myers, D.P. Olson, Central nervous system control of metabolism, Nature 2012 491:7424. 491 (2012) 357–363. https://doi.org/10.1038/nature11705.

[67] J.A.M.J.L. Janssen, New Insights into the Role of Insulin and Hypothalamic-Pituitary-Adrenal (HPA) Axis in the Metabolic Syndrome, International Journal of Molecular Sciences 2022, Vol. 23, Page 8178. 23 (2022) 8178. https://doi.org/10.3390/IJMS23158178.

[68] B. Sears, M. Perry, The role of fatty acids in insulin resistance, Lipids in Health and Disease 2015 14:1. 14 (2015) 1–9. https://doi.org/10.1186/S12944-015-0123-1.

[69] Caroline.E. Geisler, Susma. Ghimire, Chelsea. Hepler, K.E. Miller, Mark.R. Higgins, Jun. Yoshino, Samuel. Klein, Benjamin.J. Renquist, Hepatocyte Membrane Potential Regulates Serum Insulin and Insulin Sensitivity by Altering Hepatic GABA Release, BioRxiv. (2020) 475608. https://doi.org/10.1101/475608.

[70] M. Liu, R.L. Russell, L. Beigelman, R.E. Handschumacher, G. Pizzorno, β-alanine and α-fluoro-β-alanine concentrative transport in rat hepatocytes is mediated by GABA transporter GAT-2, Am J Physiol Gastrointest Liver Physiol. 276 (1999). https://doi.org/10.1152/AJPGI.1999.276.1.G206/ASSET/IMAGES/LARGE/AGIJ30117007W.JPEG.

[71] G.Y. Minuk, Gaba and hepatocellular carcinoma, Mol Cell Biochem. 207 (2000) 105–108. https://doi.org/10.1023/A:1007062802164/METRICS.

[72] C.E. Geisler, S. Ghimire, S.M. Bruggink, K.E. Miller, S.N. Weninger, J.M. Kronenfeld, J. Yoshino, S. Klein, F.A. Duca, B.J. Renquist, A critical role of hepatic GABA in the

metabolic dysfunction and hyperphagia of obesity, Cell Rep. 35 (2021) 109301. https://doi.org/10.1016/J.CELREP.2021.109301.

[73] L. Fagerberg, B.M. Hallstrom, P. Oksvold, C. Kampf, D. Djureinovic, J. Odeberg, M. Habuka, S. Tahmasebpoor, A. Danielsson, K. Edlund, A. Asplund, E. Sjostedt, E. Lundberg, C.A.K. Szigyarto, M. Skogs, J. Ottosson Takanen, H. Berling, H. Tegel, J. Mulder, P. Nilsson, J.M. Schwenk, C. Lindskog, F. Danielsson, A. Mardinoglu, A. Sivertsson, K. Von Feilitzen, M. Forsberg, M. Zwahlen, I. Olsson, S. Navani, M. Huss, J. Nielsen, F. Ponten, M. Uhlen, Analysis of the human tissue-specific expression by genome-wide integration of transcriptomics and antibody-based proteomics, Mol Cell Proteomics. 13 (2014) 397–406. https://doi.org/10.1074/MCP.M113.035600.

[74] K. Metzeler, A. Agoston, M. Gratzl, An Intrinsic γ-Aminobutyric Acid (GABA)ergic System in the Adrenal Cortex: Findings from Human and Rat Adrenal Glands and the NCI-H295R Cell Line, Endocrinology. 145 (2004) 2402–2411. https://doi.org/10.1210/EN.2003-1413.

[75] A. Mayerhofer, B. Höhne-Zell, K. Gamel-Didelon, H. Jung†, P. Redecker‡, D. Grube‡, H.F. Urbanski§, B. Gasnier‖, J.-M. Fritschy, M. Gratzl, Gamma-aminobutyric acid (GABA): a para- and/or autocrine hormone in the pituitary, The FASEB Journal. 15 (2001) 1089–1091. https://doi.org/10.1096/FSB2FJ000546FJE.

[76] H. Zemková, S.S. Stojilkovic, Neurotransmitter receptors as signaling platforms in anterior pituitary cells, Mol Cell Endocrinol. 463 (2018) 49–64. https://doi.org/10.1016/J.MCE.2017.07.003.

[77] H. Dong, M. Kumar, Y. Zhang, A. Gyulkhandanyan, Y.Y. Xiang, B. Ye, J. Perrella, A. Hyder, N. Zhang, M. Wheeler, W.Y. Lu, Q. Wang, Gamma-aminobutyric acid up- and downregulates insulin secretion from beta cells in concert with changes in glucose concentration, Diabetologia. 49 (2006) 697–705. https://doi.org/10.1007/S00125-005-0123-1.

[78] K. Harada, H. Matsuoka, H. Fujihara, Y. Ueta, Y. Yanagawa, M. Inoue, GABA signaling and neuroactive steroids in adrenal medullary chromaffin cells, Front Cell Neurosci. 10 (2016) 100. https://doi.org/10.3389/FNCEL.2016.00100/BIBTEX.

[79] A.D. and A.M. F. Cavagnini, C. Invitti, M. Pinto, C. Maraschini, A. Di Landro, Effect of acute and repeated administration of gamma aminobutyric acid (GABA) on growth hormone and prolactin secretion in man, Eur J Endocrinol. 93 (1980) 149–154.

[80] J.C. Mays, M.C. Kelly, S.L. Coon, L. Holtzclaw, M.F. Rath, M.W. Kelley, D.C. Klein, Single-cell RNA sequencing of the mammalian pineal gland identifies two pinealocyte subtypes and cell type-specific daily patterns of gene expression, PLoS One. 13 (2018) e0205883. https://doi.org/10.1371/JOURNAL.PONE.0205883.

[81] S. Patel, B. Rahmani, J. Gandhi, O. Seyam, G. Joshi, I. Reid, N.L. Smith, W.C. Waltzer, S.A. Khan, Revisiting the pineal gland: a review of calcification, masses, precocious puberty, and melatonin functions, Https://Doi-

Org.Ezproxy.Library.Wur.Nl/10.1080/00207454.2019.1692838. 130 (2020) 464–475. https://doi.org/10.1080/00207454.2019.1692838.

[82] T. Tuomi, C.L.F. Nagorny, P. Singh, H. Bennet, Q. Yu, I. Alenkvist, B. Isomaa, B. Östman, J. Söderström, A.K. Pesonen, S. Martikainen, K. Räikkönen, T. Forsén, L. Hakaste, P. Almgren, P. Storm, O. Asplund, L. Shcherbina, M. Fex, J. Fadista, A. Tengholm, N. Wierup, L. Groop, H. Mulder, Increased Melatonin Signaling Is a Risk Factor for Type 2 Diabetes, Cell Metab. 23 (2016) 1067–1077. https://doi.org/10.1016/J.CMET.2016.04.009.

[83] M. Garaulet, J. Qian, J.C. Florez, J. Arendt, R. Saxena, F.A.J.L. Scheer, Melatonin Effects on Glucose Metabolism: Time To Unlock the Controversy, Trends in Endocrinology & Metabolism. 31 (2020) 192–204. https://doi.org/10.1016/J.TEM.2019.11.011.

[84] H. Yu, S.G. Benitez, S.R. Jung, L.E. Farias Altamirano, M. Kruse, J.B. Seo, D.S. Koh, E.M. Muñoz, B. Hille, GABAergic signaling in the rat pineal gland, J Pineal Res. 61 (2016) 69–81. https://doi.org/10.1111/JPI.12328.

[85] F.P. Cappuccio, L. D'Elia, P. Strazzullo, M.A. Miller, Quantity and Quality of Sleep and Incidence of Type 2 DiabetesA systematic review and meta-analysis, Diabetes Care. 33 (2010) 414–420. https://doi.org/10.2337/DC09-1124.

[86] K. Spiegel, R. Leproult, M. L'Hermite-Balériaux, G. Copinschi, P.D. Penev, E. Van Cauter, Leptin Levels Are Dependent on Sleep Duration: Relationships with Sympathovagal Balance, Carbohydrate Regulation, Cortisol, and Thyrotropin, J Clin Endocrinol Metab. 89 (2004) 5762–5771. https://doi.org/10.1210/JC.2004-1003.

[87] J.I. Byun, Y.Y. Shin, S.E. Chung, W.C. Shin, Safety and Efficacy of Gamma-Aminobutyric Acid from Fermented Rice Germ in Patients with Insomnia Symptoms: A Randomized, Double-Blind Trial, Journal of Clinical Neurology. 14 (2018) 291–295. https://doi.org/10.3988/JCN.2018.14.3.291.

[88] A. Yamatsu, Y. Yamashita, I. Maru, J. Yang, J. Tatsuzaki, M. Kim, The Improvement of Sleep by Oral Intake of GABA and Apocynum venetum Leaf Extract, J Nutr Sci Vitaminol (Tokyo). 61 (2015) 182–187. https://doi.org/10.3177/JNSV.61.182.

Summary

Introduction

A suboptimal diet has been established as the single greatest risk factor for non-communicable diseases and is responsible for 1 in 5 premature deaths worldwide. To improve dietary quality, more emphasis could be placed on enhancing the nutritional quality of fruits and vegetables during crop selection, breeding and cultivation. While nutritional quality can be defined in terms of nutrient composition, there are also many, often unexplored, components present in our diet that might have beneficial health benefits beyond their nutritional value. Food composition databases are currently lacking information on such potentially bioactive food components and robust evidence regarding their health effects is often not available. Examples are flavonoids, anthocyanins, glucosinolates, indoles and catechins. This thesis introduces another plant component that has the potential to benefit human health: gamma-aminobutyric acid (GABA).

GABA is a non-proteinogenic amino acid and is best known as inhibitory neurotransmitter in the central nervous system. However, GABA is also present in food products. It is endogenously produced in plants like tomato, potato and melon and can also be produced during fermentation by microorganisms from its amino acid precursor glutamic acid. The GABA content can reach up to 10 grams per kilogram of product in for example matured cheeses. Interestingly, GABA receptors are not only present in the central nervous system but also in many organs and tissues throughout the body, suggesting physiologically relevant effects of peripherally present GABA. This was demonstrated in rodent studies that showed that GABA can have metabolic health effects in models for type 1 and type 2 diabetes.

Although it has been determined that GABA is taken up in the bloodstream after oral intake, it is still unclear how a food matrix affects its bioavailability and whether long-term GABA intake affects the circulating GABA concentration. Next to that, for the determination of the GABA concentration in human plasma, there is no standard, validated, analytical method available as is required for application in clinical trials. Finally, while GABA supplements are already marketed with health claims, the evidence regarding these health effects of oral GABA intake in humans is poor. This thesis therefore aimed to investigate the potential of GABA as a dietary plant component with health benefits.

Methods

Since tomato and potato have high GABA levels, while being consumed in high amounts across the world, a large collection of tomato and potato genotypes was screened for the GABA and glutamic acid levels in the fruits and tubers. Next to that, the effects of domestic cooking methods on the GABA and glutamic acid levels of tomato and potato

preparations were assessed. To proceed, a UPLC-MS/MS method was developed for the simultaneous determination of GABA and glutamic acid in human plasma and the precision and accuracy of the method was validated. The method was applied to determine the relative bioavailability of GABA from a tomato matrix, and compared to GABA dissolved in water. In a randomized crossover trial, 11 healthy men were given either a liter of pureed tomato, an equivalent amount of GABA dissolved in water, glutamic acid dissolved in water, or plain water. Blood was drawn at 19 timepoints over 24 hours, to compare plasma kinetic parameters of GABA and glutamic acid. Ultimately, the health effects of 3 months GABA supplementation were explored in 52 individuals with prediabetes in a parallel, double-blind, randomized, placebo-controlled intervention study. As the primary outcome, the effects of GABA on the postprandial glucose response were investigated. As exploratory secondary outcomes, parameters of glycemic control (HbA1c, insulin, glucagon, mean amplitude of glycemic excursions, and standard deviation as measured with flash glucose monitoring), cardiovascular health (blood pressure, 24-hour blood pressure, circulating triglycerides, cholesterol) and sleep quality were measured.

Results

The screening showed that GABA content between different potato and tomato genotypes can vary substantially. Interestingly, the GABA content remains largely constant over different harvest years, suggesting a strong genetic determinant. Specifically, the potato cv. 'Riviera' and the tomato cv. 'Madara' have GABA contents of 0.7 and 1 gram/kg, respectively. In addition, the GABA content appears to be relatively tolerant to most common domestic cooking methods, and the bioavailability of GABA from pureed tomatoes is equivalent to GABA dissolved in water. After intake, the GABA plasma concentration peaked at, on average, 75 ng/mL after 30 minutes. In fasting condition, the GABA plasma concentration was found to be 16.7 ng/mL on average and stayed relatively stable (5.8 CV%) within individuals over the course of 4 weeks.

After 3 months of GABA supplementation, the postprandial glucose response was not affected by the intervention. However, Exploratory secondary outcomes, like parameters of glycemic control and cardiovascular health, also showed no effect of GABA supplementation. However, a decrease in fasting plasma glucose and an improvement in sleep quality in individuals with prediabetes as compared to placebo was observed, although this was not significant after correction for the false discovery rate. The extent of the improvement in sleep quality seemed to be dependent on the individual's sleep quality before the intervention.

Conclusion

We showed that specific tomato and potato genotypes are potentially substantial sources of GABA in view of their large contribution to diets across the world. Furthermore, GABA is taken up into the blood as efficiently from a tomato food matrix

as from a solution in water. There are also some indications that after 3 months of supplementation, GABA might have some health benefits. GABA supplementation led to a modest reduction in fasting blood glucose and improved sleep quality in individuals with prediabetes although these were no longer significant findings after correction for the false discovery rate. Therefore, the evidence is currently not sufficient to recommend an increased dietary intake of GABA. The indications of beneficial effects that the research as presented in the current thesis provides, should be substantiated in more focused trials. Furthermore, more insight in the potential effects of GABA and their underlying mechanisms in peripheral tissues is necessary. Finally, different food products and a GABA-rich diet in general, should be used in further investigations, which should include different populations to translate the results to a real-world situation.

Acknowledgments

I would like to thank my promotor and co-promotors for teaching me how to be an independent researcher, guiding me to the right path and correcting me whenever necessary. I would like to thank Michiel for always being on my side and keeping in mind what is in my best interest. For developing the LC-MS method, I relied heavily on your expertise; thank you for patiently teaching me how to operate it and always being available for fixing errors that I did not understand. You were a very reliable supervisor and clear about the things that I could and could not expect from you. Because you were always willing to help whenever it was necessary, I felt able to confide in you whenever problems arose. I would like to thank Maarten for his outsider view, challenging ideas which I thought were standard practice. You pushed me out of my comfort zone, to think twice about concepts instead of taking the easy way out. Thank you for taking the time and investing effort in truly getting to the bottom of important topics. You also inspired me with talking about your renovation endeavors and archeological excavations, which was always entertaining during our meetings. I would like to thank Renger for his never ending contagious enthusiasm about science, which kept me motivated throughout my PhD. Of course your editing of my writing was essential for the papers that we wrote. You gave me very helpful feedback, knowing the right way to formulate that one sentence that I had been struggling with for hours. I would also like to thank you for giving me the freedom to think of my own ideas. This was scary and difficult at first, but by having the freedom to make mistakes I learned a lot and I gained a lot of self-confidence and self-knowledge.

At times, a PhD can feel like being stranded on your own little island of research. Luckily I had a lot of colleagues who were in the same "schuitje" and who help each other through the storm. I would like to thank all of them for the open atmosphere in which worries could be shared without judgment. Especially the Cornies, Merel, Lisa, Mieke, Aina, Fleur and Montse in our fancy corner office, which were my number one support group. Although we often discussed work related problems, it could also be like a good (and sometimes distracting) soap opera, with discussions about love, life and all the things that stressed us out. Also the limitless supply of cookies and candy that was present behind my desk must have relieved some stress. I will always remember my first day of work, when I stepped into the corner office for the first time and Montse immediately hugged me and gave me such a warm welcome. I would like to thank Merel for being very relatable, already during my master thesis I felt comfortable to ask you all kinds of questions. You helped me through especially the first years of my PhD, by telling me everything I needed to know and being a very good example. Mieke, I would like to thank you for being the cohesion glue of the corner office, and for being our corner office mama (although of course forever 29) with which we could spill all our problems. But also outside of the corner office you brought everyone together with some delicious bier and friet! Aina, I would like to

thank you for being super enthusiastic about everything, also about free food (is the Dutch culture getting to you?). Although there was a wall between us in the office, it was always nice to share some experiences from my PhD with you and thank you for showing me the best midnight kebabs in town! Lisa, thank you for always bringing along your discobal and the fun that comes with it. From you I also learned that drinking coffee with colleagues is one of the best parts of your PhD, especially after the COVID lockdowns this was a good way to reconnect and talk about issues also with Danny, Brecht and Frank.

Danny, thank you for our good conversations about our cultural differences, this gave me another viewpoint. You were always very considerate, inviting us to your house and bringing us gifts. You also often brightened my day with your jokes and honest opinions. Frank, thank you for the very real insights that made me think and weird associations that made me laugh. Although your many visits to our office were of course to have serious discussions with Lisa, I know it was actually to secretly steal cookies (luckily I can't eat them anymore). Brecht, thank you for the nice coffees and the times we could share our worries. Yan, thank you for always bringing a bright and happy mood and making an effort for creating a nice working atmosphere. Monique, thank you for your enthusiasm and your ability to see something funny in everything, and of course my eternal gratitude for wanting to stand beside me during my defence as my paranymph! Anouk, Thank you for sharing the burden with me during the last stretch of our PhDs. But also during my PhD you accompanied me during the PhD tour to Canada, the course in Denmark and the diabetes conference, making them a whole lot more fun with parties, good food and karaoke. Some old colleagues that left during my PhD also deserve some special thanks: Benthe, Charlotte, Xanthe, Miranda, Ian and Iris, thank you for the nice lunches and chats at the coffee machine.

I would also like to thank all the staff from the Nutrition, Metabolism and Genomics group, since I also joined the Thursday morning meetings which were very informative. Especially Lydia for her useful insights in clinical trial design and Guido for always starting the discussion and his help with the SNP assay.

I would like to thank all the colleagues from Nutritional Biology for giving feedback during the meetings in which I presented my work. Especially Suzanne, Bart, Xiaolin, Klaske, Jocelijn and Mieke during the Friday morning meetings. Also the PhD's from the NuBi group were very helpful during the monthly PhD meeting and Pol, Berber, Jacintha, Charlotte and Inge thank you for making the ESPEN congress fun! We had some nice dinners and parties. Annick, thank you for being willing to stand beside me during my defence as my paranymph. We have already been friends for a long time now, it was very nice to have someone that I can confide in working in the same group as me and I leaned on you quite a bit for support. Thank you for saving me and taking over a test day of the GO-tomaat study for a day, I wouldn't have dared to ask such a big favour of anyone else, I still owe you!

Bert and Henriette, thank you for making the cutting of a 1000 tomatoes fun and thank you Twan for your help with troubleshooting for the LC-MS. Ric, thank you for your help with writing a manuscript about a topic that is not necessarily mine. Your thorough feedback and (what seems to me) endless knowledge on the analysis of plant materials, helped in making it a good manuscript.

Henriette, thank you for all your patience in teaching me how to run a clinical trial and explaining me all the things to watch out for. Without you it would have never worked out like it did, for every problem you always had a solution. Your words: "uiteindelijk komt het altijd goed" were my mantra during difficult recruitment, screening and COVID lockdowns. When research ideas can run wild with possibilities, you thought me how to think practical. Together we have also worked out problems with tight budgets, late deliveries of food products and smoothing out my ordering mistakes. Myrthe, thank you for helping me with difficult issues of participants, I am very grateful that I could lean on your expertise as a medical doctor to assure and help worried participants. You thought me how to always look out for the well-being of the participants first. I would also like to thank Els, Anne, Rav, Mechteld, Mark, Carla and the whole human nutrition research team for their extraordinary efforts, especially during COVID times, to make these studies possible. Joao, thank you for helping me and my students make the best statistical decisions.

I would also like to thank all the students (Marit, Sanne, Eva, Suzanne, Cato, Joanne, Silke, Annika, Myrthe, Yela, Lisa and Naomi) that helped with all the practical work, you gave me the possibility to divide tasks into smaller parts which made it manageable. Special thanks for the participants in the trials, their volunteering of time, effort and motivation is what made this research possible.

Naast alle directe hulp van collega's zijn er natuurlijk ook mijn vrienden en familie die ik wil bedanken voor hun steun. Timo, bedankt voor je vertrouwen in mij, je geeft me het gevoel dat ik alles kan als ik het maar probeer. Ook al lijk ik soms een ijsklontje die alles van zich af laat glijden, jij ziet het als ik me zorgen maak en gestrest ben en je stelt me gerust. Ook was je tijdens de twee Corona jaren in mijn PhD eigenlijk mijn enige "collega" en heb je mij er doorheen geholpen met veel gezelligheid, koffietjes en lunchwandelingen.

Natuurlijk mijn ouders, de liefdevolle basis die jullie mij hebben gegeven is een privilege dat niet iedereen heeft en daar ben ik heel dankbaar voor. Jullie staan altijd voor mij klaar en hebben mij geholpen bij alle belangrijke beslissingen. Op de Emelaar kan ik bijkomen omdat de zon daar altijd schijnt, de plantjes groeien en bloeien en de schaapjes altijd vrolijk grazen in de wei. Ook Oma, Niek en Megan bedankt voor alle steun en gezelligheid tijdens de zondagmiddag lunches. Dick en Susan bedankt dat jullie mij zo hartelijk welkom hebben geheten en mij nu al voorzien van heel goed advies.

Daarnaast vooral Linda, Annick, Saskia, Yannick, Marit en Moniek, gelukkig heb ik jullie ontmoet bij SSR-W. Op jullie vriendschap kan ik vertrouwen door dik en dun, want er is al 10 jaar lang altijd plaats voor een toetje. Door de prikkelende spelletjesavonden, diners met veel ui, feestjes, weekenden met zachte een-bultige kamelen, vakanties in de natuur of met Edje en festivals kan ik alles aan.

About the author

Tessa De Bie was born on the 29th of April 1993 in Utrecht, The Netherlands. In 2005 she started secondary school at the "VMBO" level, to transfer to "HAVO" in the second year and transfer to "VWO" after the fifth year with extra courses in chemistry and biology to be able to enroll in the bachelor Nutrition and Health at the Wageningen University in 2012. In her bachelor she focused on courses in immunology and microbiology and wrote a thesis about the epigenetic effects of folate in early life. In 2015 she proceeded with the master Nutrition and Health with the specialization Molecular Nutrition and Toxicology with a master thesis at the chairgroup Nutrition, Metabolism and Genomics and internships at "het Nationaal Schoolontbijt" and BioActor in Maastricht.

After graduating, she started in April 2018 at the Division of Human Nutrition and Health with the PhD project that is described in this thesis. During the project she was supervised by Maarten Jongsma from Wageningen Plant Research and Michiel Balvers and Renger Witkamp from the Nutritional Biology group. The research was funded as a TKI project with support from Nunhems Netherland, Agrico Research and Avebe. During the project she developed an LC-MS method, and set-up and performed two clinical trials. She supervised 10 master students during their thesis and contributed to teaching within several courses. During her time at the department she was also active as a member of the PhD committee.

List of publications

This thesis

de Bie, T. H., Balvers, M. G., de Vos, R. C., Witkamp, R. F., & Jongsma, M. A. (2022). The influence of a tomato food matrix on the bioavailability and plasma kinetics of oral gamma-aminobutyric acid (GABA) and its precursor glutamate in healthy men. Food & Function, 13(16), 8399-8410.

de Bie, T. H., Witkamp, R. F., Jongsma, M. A., & Balvers, M. G. (2021). Development and validation of a UPLC-MS/MS method for the simultaneous determination of gamma-aminobutyric acid and glutamic acid in human plasma. Journal of Chromatography B, 1164, 122519.

Other publications

Stevens, Y., de Bie, T., Pinheiro, I., Elizalde, M., Masclee, A., & Jonkers, D. (2022). The effects of citrus flavonoids and their metabolites on immune-mediated intestinal barrier disruption using an in vitro co-culture model. British Journal of Nutrition, 128(10), 1917-1926.

de Bie, T.H. & Koning, R. (2017). Beating Arthritis with Dietary Supplements: Can This Be True? Agro FOOD Industry Hi Tech, 28, 5.

Overview of completed training activities

Discipline specific activities

Name of the course/meeting	*Organizer*	*Country*	*Year*
Symposium Diabetesacademie	Diabetesacademie	NL	2018
Food for future symposium	WUR	NL	2018
Annual Dutch Diabetes Research Meeting	NVDO	NL	2018
Young NVDO Meeting	NVDO	NL	2019
Diabetes summer school	DDA	DK	2019
Nutrition 2021	ASN	VS	2021
Vascular Biology and Pathology	Hartstichting	NL	2021
Annual Dutch Diabetes Research Meeting	NVDO	NL	2022
ESPEN 2022	ESPEN	AU	2022
Nutritional Science Days	NAV	NL	2022

General courses

Name of the course	*Organizer*	*Country*	*Year*
VLAG PhD week	VLAG	NL	2018
Workshop carousel	WGS	NL	2018
Start to supervise thesis students	WGS	NL	2019
Introduction to R	VLAG	NL	2019
Supervising of thesis students	WGS	NL	2019
Applied statistics	VLAG	NL	2019
Educational inspirational meetings	EduSupport	NL	2018
Young NAV meets ENLP: Nutritional Leadership Workshop – Debatteren	NAV	NL	2019
Philosophy and Ethics of Food Science and Technology	VLAG	NL	2020
GCP cursus	Profess	NL	2019
COMPLEAT-training	HRU Karen Borgonjen	NL	2019

Other activities

Name of the course	*Organizer*	*Country*	*Year*
Preparation of research proposal	HNH	NL	2019
PhD study tour to Canada	HNH	CA	2019
Weekly group meetings	HNH	NL	2019-2022
NuBi Journal club and lectures	HNH	NL	2019-2022
Divisional seminars	HNH	NL	2019-2022

The research described in this thesis was financially supported by a consortium of companies (Agrico Research, Nunhems Netherlands, AVEBE) taking part in a public-private partnership under the "Topsector Agri-Food" program supported by the Dutch Ministry of Economic Affairs [grant number AF17048].

Financial support from Wageningen University for printing this thesis is gratefully acknowledged.

Cover design by Tessa De Bie, Midjourney

Printed by ProefschriftMaken || www.proefschriftmaken.nl